BIOSYNTHESIS OF ENTEROBACTERIAL COMMON ANTIGEN, THE ECA-TRACE PHENOTYPE OF SALMONELLA TYPHIMURIUM AND THE ROLE OF THE RFE GENE IN 08 SIDE-CHAIN SYNTHESIS IN ESCHERICHIA COLI

1993

HEINE

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Biosynthesis of Enterobacterial Common Antigen.

The ECA-Trace Phenotype of

Salmonella typhimurium and The Role of the

rfe Gene in O8 Side-Chain Synthesis in *E. coli*

by

Henry Simpers Heine III

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the Faculty of the Department of Microbiology

Graduate Program of the Uniform Services University of the

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Doctor of Philosophy, 1993

ABSTRACT

Title of Dissertation:

Biosynthesis of Enterobacterial Common Antigen.The ECA-Trace Phenotype of *Salmonella typhimurium* and The Role of the *rfe* Gene in O8 Side-Chain Synthesis in *E. coli*

Henry S. Heine: Candidate, Doctor of Philosophy, 1993

Dissertation directed by: Paul D. Rick, Ph.D., Professor, Department of Microbiology.

Enterobacterial Common Antigen (ECA) is an outer membrane glycolipid synthesized by all members of the Enterobacteriaceae. The carbohydrate portion of ECA consists of linear heteropolysaccharide chains comprised of trisaccharide repeat units having the structure ->3)-α-D-Fuc4NAc-(1->4)-β-D-ManNAcA-(1->4)-α-D-GlcNAc-(1->. The trisaccharide repeat unit is synthesized via the sequential transfer to undecaprenol monophosphate of GlcNAc-1-phosphate, ManNAcA, and Fuc4NAc from UDP-GlcNAc, UDP-ManNAcA, and TDP-Fuc4NAc, respectively. In *Salmonella typhimurium* the genes involved in the synthesis of ECA are located at two regions on the chromosome- the *rfe/rff* and *rfb* regions. *Salmonella typhimurium* mutants possessing *rfb* lesions (specifically the *rfb* A and B genes) accumulate ManNAcA-GlcNAc-pyrophosphoryl-undecaprenol (lipid II), the dissacharide-

linked lipid precursor of ECA, in the inner membrane. These mutants appear to synthesize "trace" amounts of ECA, and have increased permeability to SDS and other hydrophobic agents. The "ECA-trace" mutants are unstable, readily acquiring secondary mutations in the *rfe* or *rff* loci.

Data were obtained which indicated that the sugar composition of ECA synthesized by "ECA-trace" mutants was identical to wild-type ECA. In addition, analyses of isolated cytoplasmic and outer membrane fractions obtained from "ECA-trace" mutants and wild type cells revealed that the trace amounts of ECA, as well as wild-type ECA, are localized in the cytoplasmic membrane.

The *rffT* locus encodes the structural gene for the Fuc4NAc transferase involved in ECA synthesis. Introduction of a mutation in *rffT* into *S. typhimurium* resulted in a mutant strain which accumulated lipid II in the inner membrane but was fully ECA-minus. In addition, this strain still retained increased sensitivity to SDS. These data support the conclusion that the accumulation of lipid II but not the presence of trace ECA is in some way responsible for the increased sensitivity of "ECA-trace" mutants to hydrophobic agents.

Lipid II was purified, from an "ECA-trace" strain and its structure was found to be ManAcA-GlcNAc-pyrophosphorylundecaprenol as determined by FAB-tandem MS/MS. In addition, two ManAcA-GlcNAc-pyrophosphoryl-diglycerides (lipids IIa and IIb) were identified. The structures of these lipids appeared to differ only with respect to the fatty acyl subsituents located in the <u>sn</u>-2 position of the glycerol.

Additional studies were directed toward determining the role of the *rfe* gene in the synthesis of O8-side chains in *E. coli* O8. The synthesis of O8 side-chains was shown to be sensitive to tunicamycin. Carbohydrate analysis of purified O8 side-chains using a Dionex BioLC carbohydrate analyzer system showed the terminal reducing sugar to be N-acetyl-glucosamine. This provided evidence that GlcNAc-pyrophosphorylundecaprenol (lipid I), the first intermediate of ECA synthesis is also an intermediate in the synthesis of O8 side-chains.

DEDICATION

This work is dedicated to three gentleman who had great influence in my life and unfortunately passed away during my graduate tenure and could not see its completion.

My grandfather; Haynes Monroe Pridgen

My Uncle; Haynes Pridgen Jr.

Dr. John Hanks

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My gratitude and appreciation to:

To my wife Mary Ann and my children Shannon and Charles who have made the real sacrifices while I have pursued my folly.

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My mother, Barbara Heine-Redmond.

My brother, Clint Heine.

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To each one who extended a hand of friendship or advice when I needed it most.

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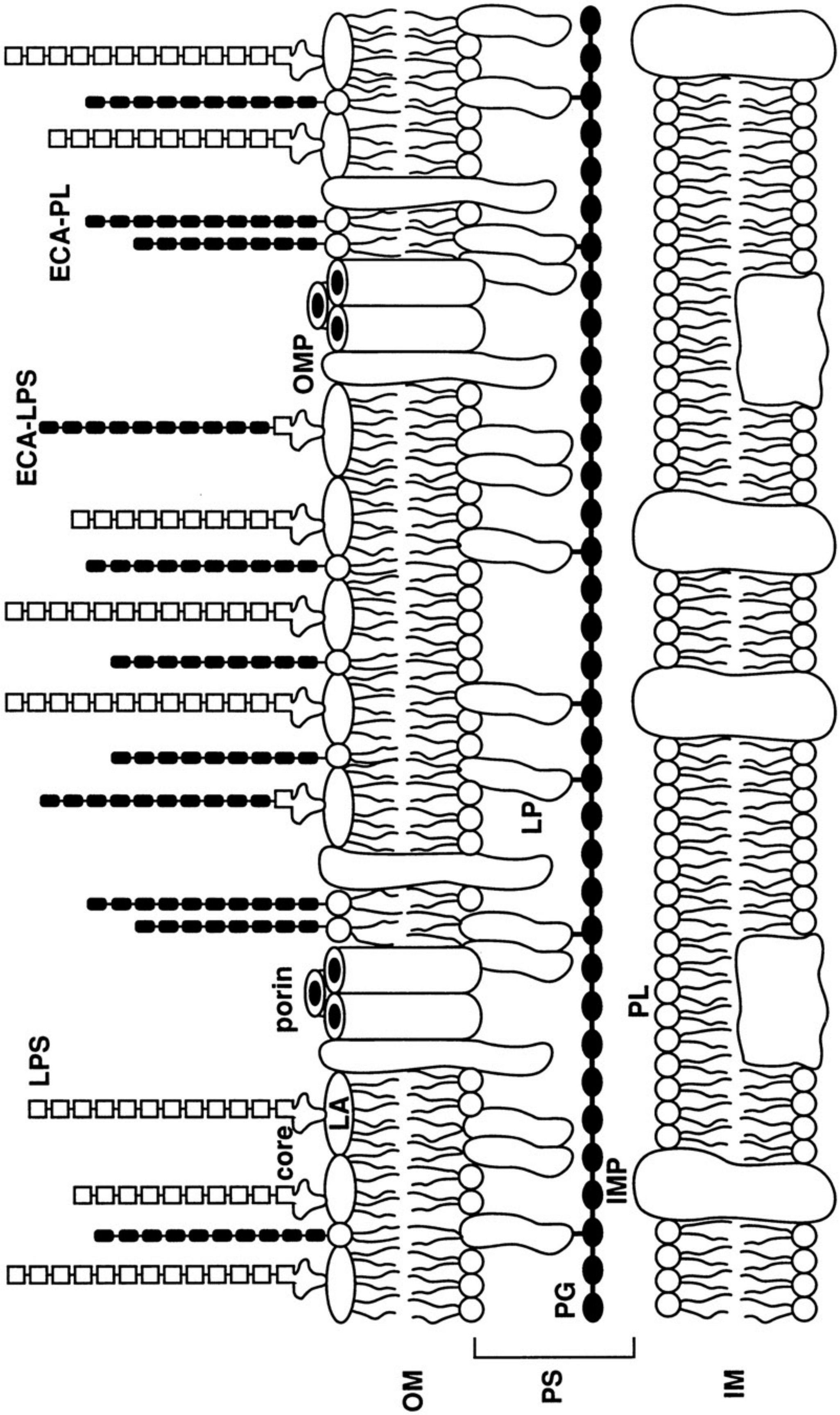
INTRODUCTION

Gram-negative bacteria are resistant to a wide variety of antibacterial agents and degradative enzymes (Inouye, 1979). This is in large part due to the "barrier" properties of the gram-negative bacterial cell envelope the structure of which is shown in Figure 1. The cell envelope is comprised of two distinct membrane systems which sandwich a single peptidoglycan layer. The region between the two membranes is called the periplasmic space (Glauert and Thornley, 1969). The two membranes are referred to as the cytoplasmic or inner membrane and the outer membrane. It is the outer membrane which is responsible for most of the "barrier" or resistance properties of gram-negative bacteria. This membrane serves as both a containment for the contents of the periplasmic space and as a selective diffusion barrier (Inouye, 1979).

The outer membrane is an asymmetrical bilayer containing three major components; phospholipid, protein, and a unique glycolipid, lipopolysaccharide (LPS). In *Escherichia coli* the outer membrane is anchored to the peptidoglycan layer by a lipoprotein component, "murein lipoprotein". The C-terminus of the lipoprotein is covalently attached to 10-12% of the available diaminopimelate residues of the peptidoglycan layer. The murein lipoprotein contains three fatty acid residues attached to the N-terminus. These residues serve to anchor it in the outer membrane (Braun and Rehn, 1969). Related lipoproteins have also been identified in other

Figure 1. Structure of the Cell Envelope of Gram-Negative Bacteria (Enterobacteriaceae). A schematic representation of the structure of the cell envelope. OM, outer membrane; IM, inner membrane; PS, periplasmic space; PG, peptidoglycan (murein); IMP, inner membrane protein; PL, phospholipid; LP, lipoprotein (note bound and free forms); OMP, outer membrane protein including porins; LPS, lipopolysaccharide; LA, lipid A; core, core region of LPS. O-antigen oligosaccharide repeat units are represented with open boxes. ECA-PL, enterobacterial common antigen phospholipid bound form (haptenic). ECA-LPS, enterobacterial common antigen lipopolysaccharide bound form (immunogenic). ECA oligosaccharide repeat units are represented with solid ovals. Note, that in certain strains ECA can be attached to the LPS core in place of O-antigen. Adapted from Lugtenberg, B., and L. van Alphen. 1983.

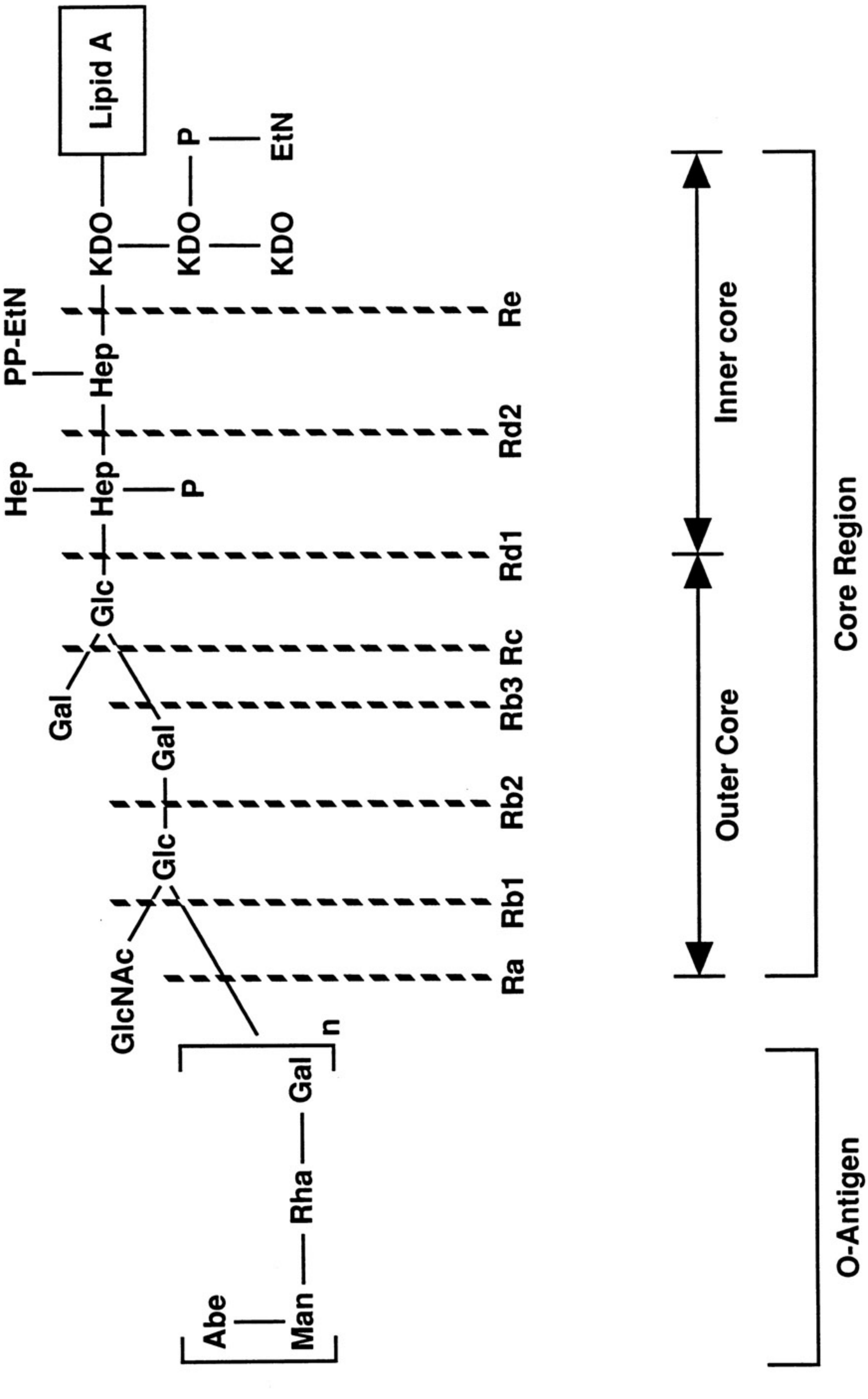

OM
PS
IM
LPS
core
LA
porin
ECA-LPS
OMP
ECA-PL
PG
IMP
LP
PL

members of the family Enterobacteriaceae as well as in other gram negative bacteria (Nakamura and Inouye, 1979; Mizuno and Kageyama, 1978).

Many of the remaining outer membrane proteins seem to be involved in specific or non-specific transport. For example, the "porins" are a group of outer membrane proteins that span the outer membrane and contain channels that allow either nonspecific transport of small hydrophilic molecules (less than 600 daltons) or specific transport of various sized compounds. Outer membrane proteins involved in specific transport such as maltose and maltodextrins (LamB), nucleosides (Tsx), vitamins (BtuB), iron (FepA), and several others have also been identified. In addition a few lipase and protease activities have been observed (Nikaido and Vaara, 1985).

The outer membrane lipid bilayer asymmetry is due to the topological distribution of LPS and phospholipid. LPS may occupy up to 50% of the outer leaflet surface area of the outer membrane with the remaining space taken up by protein. The structure of LPS, shown in Figure 2, is well understood for *Salmonella typhimurium*. LPS is composed of a lipid portion "lipid A", a "core" oligosaccharide region, and the "O" antigen polysaccharide. The lipid A portion is a phosphorylated glucosamine disaccharide in which the two amino-sugars are linked ß-(1-6). In addition, the lipid A disaccharide is substituted with up to seven fatty acids residues (Takayama et al., 1983). The core region can be divided into two regions, the "inner" core and the "outer" core, based on sugar composition and degree of variability among species. The inner core varies little from species to species and contains 3-deoxy-D-mannooctulosonic acid (KDO) and L-glycero-D-mannoheptose linked to lipid A. Ethanolamine and phosphate groups are also present which, along with the KDO residues and lipid A, yield a large number of negatively

Figure 2. Lipopolysaccharide Structure. A schematic representation of the structure of the LPS from *S. typhimurium.* Substructures are indicated and chemotypes of mutant LPS forms are indicated by broken lines. Abbreviations: Abe, abequose: Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; Glc, D-glucose; Hep, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-mannooctulosonic acid; EtN, ethanolamine; P, phosphate. (Rick, 1987).


Lipid A
KDO
KDO
KDO
P
EtN
PP-EtN
Hep
Hep
Hep
Hep
P
Glc
Gal
Gal
Glc
GlcNAc
Abe
Man
Rha
Gal
n
Re
Rd2
Rd1
Rc
Rb3
Rb2
Rb1
Ra
Inner core
Outer Core
Core Region
O-Antigen

charged groups (Galanos et al., 1977; Luderitz et al., 1971). The outer core region is more variable and usually contains uncharged hexoses and hexosamines. The O-antigen is attached to the outer core and it is structurally the most variable region (Luderitz et al., 1971). Mutations affecting the synthesis of the O-antigen or outer core region of LPS result in structurally incomplete forms collectively referred to as "rough" LPS molecules. Mutations that affect the synthesis of the inner core region result in "deep-rough" LPS molecules. The rough or "R" forms are classified according to the structure or "chemotype" of the incomplete LPS molecule (Fig 2).

Phosphatidylethanolamine (PE) is the dominant phospholipid in the outer membrane, and it accounts for approximately 80% of the total OM phospholipid. Phosphatidylglycerol (PG) is also present along with trace amounts of cardiolipin (CL); these two phospholipids account for approximately 19% and 1% of the OM phospholipid, respectively (Osborn et al., 1972). The head groups of outer membrane phospholipids in intact cells of *S. typhimurium* are resistant to phospholipase and cannot be crosslink with cyanogen bromide-activated dextran. This suggests that the phospholipid in such strains is absent from the outer leaflet of the outer membrane (Kamio and Nikaido, 1976). Spin-labeling techniques and X-ray diffraction studies also suggest that the phospholipid in the outer membrane exists as a monolayer (Nikaido et al., 1977; Emmerling et al., 1977). The inner leaflet contains phospholipid and protein, whereas LPS is absent (Smit et al., 1975). Additional evidence for the asymmetrical distribution of outer membrane components has been obtained from immunoelectron microscopy using anti-LPS antibodies. These studies showed that LPS was only present in the outer leaflet (Muhlradt and Golecki, 1975; Mulford and Osborn, 1983).

Phospholipid bilayers are permeable to hydrophobic compounds. In contrast, the outer membrane of gram-negative bacteria functions as a permeability barrier to these compounds. The lipid A and inner core regions of LPS are very negatively charged, due to phosphate and KDO residues. As a consequence, LPS forms complexes with inorganic cations and polyamines to form a rigid molecular structure in the outer membrane(Schindler and Osborn, 1979; Galanos and Luderitz, 1975; Nikaido and Vaara, 1985). EDTA treatment of intact *E. coli* and *S. typhimurium* results in release of LPS from the outer membrane and renders these strains more sensitive to hydrophobic agents (Leive, 1965; Leive, 1974; Nikaido and Vaara, 1985). This treatment also facilitates lysozyme access to the peptidoglycan layer. Thus it is the structure and chemical nature of LPS, along with the outer membrane proteins and the absence of phospholipid from the outer leaflet, that gives the outer membrane its selective permeability properties.

Mutations which result in a deep rough LPS show altered permeability to hydrophobic agents, with little or no change in the ability of hydrophilic agents to diffuse through the outer membrane porins (Nikaido, 1976; Roantree et al., 1977; Sanderson et al., 1974; Schlecht and Schmidt, 1969). More specifically, this is true only for mutations that result in the synthesis of a deep-rough LPS having a Rd_1-, Rd_2-, or Re-chemotype. A key characteristic of these mutants is the reduction of total outer membrane protein content with a compensating increase in phospholipid content; in contrast, the total LPS content remains unchanged or slightly increased (Ames et al., 1974; Smit et al., 1975). Accordingly, these mutants contain phosphatidyl-ethanolamine that is accessible to phospholipase C and cyanogen bromide activated dextran, thus providing evidence of the presence of PE in the outer leaflet of the outer membrane (Kamio and

Nikaido, 1976). This increase in the phospholipid content is believed to reflect the occurrence of phospholipid bilayer regions which allows the diffusion of hydrophobic agents across the outer membrane.

Two other LPS mutations which affect the sensitivity of gram-negative bacteria to hydrophobic agents are mutations in the *rfaP* gene of *S. minnesota*, *E. coli* and *S. typhimurium* and mutations in the *nbsB* gene of *E. coli.* The *rfaP* mutants lack phosphate in the inner core region. These mutants are sensitive to hydrophobic agents and detergents (Schlecht and Schmidt, 1969; Schlecht and Westphal; 1970, Sukupolvi and Vaara, 1989). Mutations in the *nbsB* gene result in a heterogeneous LPS (mixed chemotypes) and such mutants are sensitive to several hydrophobic agents (Coleman and Leive, 1979).

A final class of LPS-related permeability defects are those that result from mutations in the *sfr* gene of *E. coli* and the *rfaH* gene of *S. typhimurium.* These genes are related to "sex factor regulation" but also seem to produce heterogeneous LPS forms due to reduction of glycosyl-transferase activities (Creeger et al., 1984; Lindberg and Hellerquist, 1980).

With a few exceptions, mutations which result in loss of outer membrane proteins do not affect sensitivities to hydrophobic agents. However, in some cases they actually cause an increase resistance to hydrophilic agents. In the case of the ompA protein and porin proteins, the loss of one is compensated for by increases in other outer membrane proteins (Chai and Foulds, 1977; Van Alphen et al., 1977). In mutations where increased sensitivity to hydrophobic agents was observed, either a decrease in total outer membrane protein was implicated (Sukupolvi and Vaara, 1989) or a defective form was inserted into the membrane causing

disruption of normal interactions between outer membrane components (Misra and Benson, 1988; Sukupolvi and Vaara, 1989).

Mutations which preclude lipoprotein synthesis also result in sensitivity to a few hydrophobic agents and leakage of periplasmic contents. These effects have been observed for the *lpo* mutation of *E. coli* which abolishes lipoprotein synthesis, and the *lkyD* mutation of *S. typhimurium* which reduces the bound-form of lipoprotein (Hirota et al., 1977; Chai and Foulds, 1977; Weigand and Rothfield, 1976). These mutants show changes in morphology and "blebbing" of the outer membrane. The decrease in lipoprotein-mediated peptidoglycan linkage to the outer membrane is thought to lead to a breakdown in the structural integrity of the envelope thus resulting in altered outer membrane permeability (Sukupolvi and Vaara, 1989).

A phospholipid mutation in *E. coli* has been described which yields a decrease in the synthesis of phosphatidylethanolamine and results in increased sensitivity to hydrophobic agents (Raetz and Foulds, 1977). This is the only defect in phospholipid synthesis to date that shows this effect. The reason for this defect is not known since the composition of the outer membrane in this mutant has not been investigated. Since phosphatidylethanolamine is the predominant phospholipid in the outer membrane, it has been suggested that a decreased phosphatidylethanolamine content could lead to poor interactions between remaining outer membrane components (Raetz, 1986; Sukupolvi and Vaara, 1989)

There are several other mutations of *E. coli* and *S. typhimurium* that result in altered permeability to hydrophobic compounds; these include the *abs, acrA, envA, envC* and the Δ*rfb* mutations.

The outer membrane of *E. coli abs* mutants have a normal LPS, phospholipid and protein composition. However, an increase in amino group content of the LPS was reported (Clark, 1984). It has been suggested that this may cause a decrease in the net negative charge of the LPS thereby resulting in a reduced LPS-LPS interactions due to concomitant loss of cations leading to a disruption of the outer membrane.

Little is known about the specific defect caused by the *acrA* mutation in *E. coli*. Reports on the envelope composition of this mutant have been contradictory (Sukupolvi and Vaara, 1989). Both inner membrane alterations (Nakamura and Suganuma, 1972) and a reduced Lipid A phosphate content (Coleman and Leive, 1979) have been suggested to result from mutations in *acrA*; however, subsequent studies do not support these conclusions (Leive et al., 1984).

The *envA* and *envC* mutations are phenotypically related in that they both affect cell division and result in the development of chains of bacteria due to incomplete septa formation (Normark et al., 1969; Normark et al., 1971; Rodolakis et al., 1973). The *envC* mutant may be defective in phospholipid turnover function, and shows changes in phospholipid content (Michel, 1979; Michel et al., 1977). This mutant also has phospholipase C sensitive phospholipids (Starkova et al., 1981). Lipid bilayers areas may be present in the outer leaflet of the outer membrane but experiments to verify this have not been reported.

The *envA* mutation has been studied in more detail. In addition to this mutant's sensitivity to a variety of hydrophobic agents, the murein layer of these mutants has been shown to be sensitive to lysozyme in the absence of EDTA (Normark et al., 1971). The *envA* mutant has recently been shown to have a leaky phenotype, releasing several periplasmic enzymes (Young and

Silver, 1991). The mutant has reduced amounts of LPS in the outer membrane, while protein and phospholipid content remain unchanged. Qualitative differences in LPS, protein and phospholipid have not been observed. The hydrophobic sensitivity phenotype can be reversed by a second mutation, *sefA*. This gene is involved in septa formation and the mutation results in a quantitative increase in outer membrane protein content while LPS and phospholipid levels remain unchanged (Grundstrom et al., 1980). The *envA* gene codes for the N-deacetylase which is required to remove the acetyl group from UDP-3-monoacyl-GlcNAc; the second step in lipid A synthesis (Christian Raetz: personal communication). At the very least this would result in an altered lipid A lacking two to four fatty acid residues in the final molecule as N-acylation would be blocked. This assumes that the remaining biosynthetic enzymes are able to react with the altered molecule. It seems more likely that another N-deacetylase activity is substituted in the overall biosynthesis or the *envA* mutation is leaky enough to allow some "wild-type" Lipid A synthesis. This would account for the observation that the LPS did not show any qualitative changes in *envA* mutants (Young and Silver, 1991). The substitution of another N-deacetylase activity might alter the kinetics of lipid A biosynthesis. The observed outer membrane disruption of *envA* mutants could be the result of altered lipid A biosynthesis kinetics or an altered molecule.

A deletion of the *his-rfb* (Δ*rfb*) region in *S. typhimurium* results in the sensitivity of such mutants to hydrophobic compounds. The *rfb* locus codes for O-antigen biosynthetic enzymes, and in *S. typhimurium* two of these genes (*rfbA* and *rfbB*) are also involved in the biosynthesis of enterobacterial common antigen or ECA (Mäkelä and Mayer, 1974; Lew et al., 1986). The Δ*rfb* mutants are still able to make "trace" amounts of ECA as

determined by Western blots using an anti-ECA monoclonal antibody. They also accumulate a lipid-linked intermediate of ECA (Rick et al., 1988). These mutants were shown to have qualitative sensitivities as compared to wild type and and known sensitive mutants to several hydrophobic agents, most notably detergents such as sodium dodecylsulfate (SDS) (Mäkelä et al., 1976). The reduced amount of ECA and/or the accumulation of the lipid-linked intermediate are believed to be involved in the sensitivity of these mutants to hydrophobic agents.

ECA is a cell envelope component found in all members of the Enterobacteriaceae. ECA was originally discovered by Kunin (Kunin et al., 1962). Kunin, using a passive hemagglutination assay incubated rabbit antisera raised against whole heat-killed cells obtained from145 different *E. coli* O-antigen serotypes with erythrocytes coated with extracts from those same serotypes. It was discovered that a few antisera gave a positive agglutination reaction for all serotypes. The most notable was antisera raised against *E. coli* O14. This antisera was also shown to agglutinate erythrocytes coated with extracts from many other members of the Enterobacteriaceae including *Shigella, Klebsiella, Proteus, Erwinea* and *Salmonella*. Other gram-negative bacteria not belonging to the Enterobacteriaceae, as well as a few gram-positive species, were also tested and found to be ECA negative (Kunin et al., 1962). Additional taxonomic surveys have established the uniqueness of ECA to the Enterobacteriaceae (Le Minor et al., 1972; Maeland and Digranes, 1975; Whang and Neter, 1973). Accordingly, it has been proposed that ECA could serve as a key taxonomic factor in the classification of the Enterobacteriaceae (Ramia et al., 1982).

Compared to LPS, ECA is a relatively minor component of the cell envelope; there are approximately 4×10^5 molecules of ECA per cell whereas

there are $2x10^6$ molecules per cell of LPS (Meier-Dieter et al., 1989). The carbohydrate portion consists of repeating trisaccharide units containing N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc). The sugars are linked ->3)-α-D-Fuc4NAc-(1->4)-β-D-ManNAcA-(1->4)-α-D-GlcNAc-(1-> (Mannel and Mayer, 1978; Lugowski et al., 1983). The number of repeat units range from 12-40 based on the observed molecular weight range of 12,000 to 35,000 daltons (Rick et al., 1985).

The carbohydrate portion is anchored to the membrane via a phospholipid in all Enterobacteriaceae. This lipid has been identified as L-α-phosphatidic acid. The products released by mild acid hydrolysis and cleavage by phospholipase D indicate that the carbohydrate portion is linked to the lipid via the phosphate group at the C-3 position of the glyceride moiety. The fatty acids have been identified as a 16:0 attached to the C-1 position and a 16:1 attached to C-2. Lessor amounts of 16:0, 18:0, or 18:1 may also be substituted in the C-2 position (Kuhn et al., 1988).

In addition to the phospholipid-linked ECA, certain strains and species simultaneously possess an LPS-linked form. This form is only found in strains which synthesize a LPS having a complete K-12, R1 or R4 core structure but lacking O-side chains. Only 5% of the total available LPS is linked to ECA in strains possessing this form. (Schmidt et al., 1976; Whang et al., 1982; Kuhn and Mayer, 1987; Meier et al., 1986).

It was the LPS-linked ECA form in whole heat-killed cells of *E. coli* O14 that elicited the antibodies which led to the original discovery of ECA by Kunin. Anti-ECA antibodies were not elicited when rabbits were immunized with whole heat-killed cells of strains that only possessed the phospholipid-linked form of ECA. The original immunological observations led to the

nomenclature of ECA "immunogenic" and "haptenic" forms for the LPS-linked and phospholipid-linked forms, respectively. It was not until the structure and nature of these forms were understood that the immunolgical observations could be explained. Cell-free preparations of the "haptenic" form are also capable of eliciting a humoral immune response; however, this form of ECA (ECA-PL) probably requires a co-immunity factor to render it immunogenic (Kuhn et al., 1981; Lugowski et al., 1983) while the LPS core serves that function in the LPS-linked forms.

A cyclic form of ECA, lacking any lipid component, has also been reported in *Shigella sonnei* "phase I" cells. This form is composed of 4-6 repeat units (Dell et al., 1984). However this form has not been detected in other organisms, and it has been suggested that this observation may be an artifact of the extraction procedure (Kuhn et al., 1988).

The biosynthesis of ECA is believed to occur sequentially in the cytoplasmic membrane via transfer of sugars from nucleotide carriers to a unique carrier lipid. The first step involves the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to undecaprenyl phosphate to form GlcNAc-pyrophosphorylundecaprenol (lipid I) (Rick et al., 1985; Barr and Rick, 1987). This intermediate was originally identified as a nonpolar lipid that could be labeled with [^{3}H]GlcNAc. The component was identified as lipid I by qualitative analysis of the acid released carbohydrate, using chromatography and $NaBH_4$ reduction. The intact lipid was also compared with the chromatographic mobility of a GlcNAc-pyrophosphorylundecaprenol standard, and found to co-migrate in two chromatography systems (Rick et al., 1985).

An important tool for the elucidation of the ECA pathway has been the antibiotic tunicamycin. Tunicamycin is a specific inhibitor of enzymes that

catalyze the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to polyprenol phosphate acceptors (Takatsuki et al., 1975; Tkacz and Lampen, 1975; Mahoney and Duskin, 1979). ECA biosynthesis, and more specifically lipid I synthesis, have been shown to be inhibited by tunicamycin (Rick et al., 1985; Barr and Rick, 1987).

The relationship of lipid I to ECA biosynthesis was established in several ways. Both [^{3}H]GlcNAc incorporation into lipid I and the synthesis of ECA were shown to be abolished when cells were grown in the presence of tunicamycin (Rick et al., 1985). In addition, ECA-minus mutants of the *rfe* class were unable to incorporate [^{3}H]GlcNAc into lipid I in vitro (Rick et al., 1985; Meier-Dieter et al., 1990). Further, pulse-chase experiments using whole cells revealed that [^{3}H]GlcNAc-labeled lipid I was chased into the ECA polymer after the addition of nonradioactive GlcNAc (Rick et al., 1985).

The next step in ECA synthesis is the transfer of ManNAcA from UDP-ManNAcA to lipid I to form ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid II). This step was demonstrated in an in-vitro system using *E. coli* membranes (Barr and Rick, 1987). Lipid II has also been shown to accumulate in Δ*rfb* mutants of *S. typhimurium* since these mutants are unable to synthesize the nucleotide sugar, TDP-Fuc4NAc, the donor of Fuc4NAc residues in ECA synthesis. The accumulation of lipid II in these mutants could be abolished with tunicamycin (Rick et al., 1988).

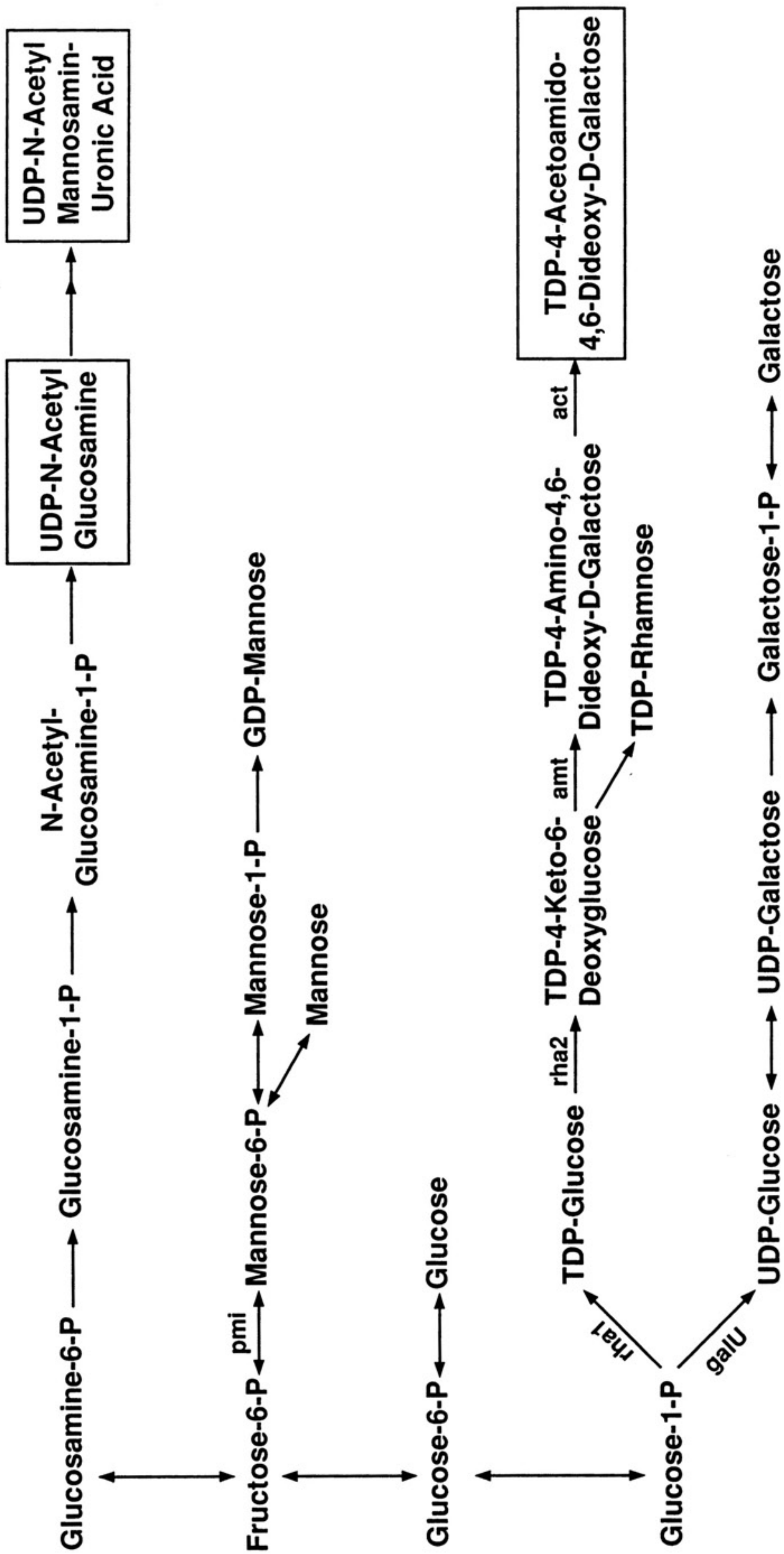
The final step in the synthesis of the ECA trisaccharide repeat unit is the transfer of Fuc4NAc from TDP-Fuc4NAc to lipid II to form lipid III. The overall reaction is inhibited by tunicamycin, and de novo synthesis of lipid III in vitro is precluded by omission of either UDP-GlcNAc or UDP-ManNAcA from reaction mixtures (Barr et al., 1989). The final steps in ECA biosynthesis; i.e., polymerization, transfer of the polysaccharide to

phospholipid or LPS, and translocation, have yet to be determined. The pathways for synthesis of nucleotide sugars involved in ECA synthesis and the overall biosynthetic pathway of ECA as they are currently understood are summarized in Figures 3 and 4.

The genetics of ECA are complex since the products of many of the genes involved in ECA synthesis are also involved in the synthesis of other polymers in some members of the Enterobacteriaceae (Mäkelä and Mayer, 1974). ECA genetics have been best characterized in *Salmonella* and *E. coli* (Mäkelä et al., 1976). In all species thus far examined, the *rfe-rff* loci have been shown to be required for ECA biosynthesis. This region is located at 85 minutes on the *E. coli* chromosome and at 84 minutes on the *S. typhimurium* chromosome between the *met* and *ilv* genes. In a few species some of the ECA biosynthetic genes have been relocated to other regions of the chromosome (Mäkelä and Mayer, 1974). Specific examples are the *rfbA* and *B* of *S. typhimurium* and unidentified genes in *E. coli* O14:K7.

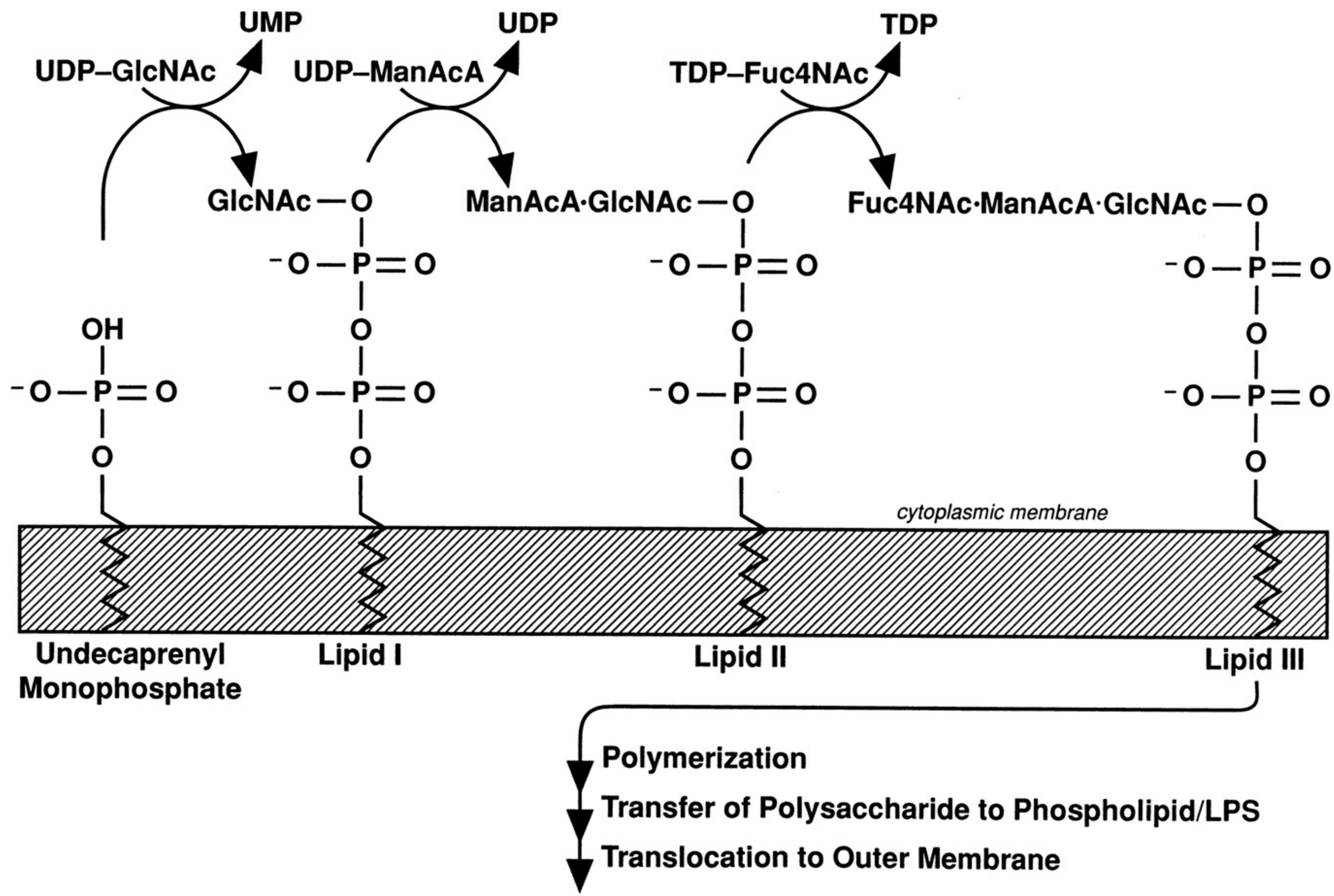
The *rfb* region contains the genes for the enzymes involved in the biosynthesis of the O-antigen for each species. The role of the *rfb* region in ECA biosynthesis of *S. typhimurium* was discovered by the analysis of recombinants in which the *rfe-rff* genes of *S. typhimurium* replaced the *rfe-rff* region of other species such as *Salmonella montevideo* (Mäkelä et al., 1974; Mäkelä and Mayer., 1974). At the time of these original studies the chemical nature of ECA was not known nor had the *rff* region adjoining the *rfe* locus been identified. It was observed that the *rfe*(*rff*) region of *S. typhimurium* would support only reduced amounts of ECA synthesis in other *Salmonella* species. In contrast, reciprocal crosses transfering *Salmonella montevideo* or *E. coli rfe*(*rff*) regions into *S. typhimurium* would support ECA synthesis in *S. typhimurium* regardless of the status of

Figure 3. Nucleotide-Sugar Pathways Relative to ECA Biosynthesis. Nucleotide-sugars directly involved in ECA synthesis are boxed. Other carbohydrate pathways shown are relevant to strain constructions, O8 O-antigen synthesis and LPS (see text). All carbohydrates are in the D-configuration, with the exception of TDP-L-Rhamnose. Relevant enzyme abbreviations are: pmi, phosphomannoisomerase; pgi, phosphoglucoisomerase; galU, uridine diphosphoglucose pyrophosphorylase; rha1, thymidine diphosphoglucose pyrophosphorylase; rha2, thymidine diphosphoglucose oxidoreductase; amt, TDP-4keto-6-deoxy-D-glucose transaminase; act, TDP-4-amino-4,6-dideoxy-D-galactose transacetylase. (Adapted from Gabriel, O., 1987 and Nikaido et al., 1966.)



Glucosamine-6-P
Glucosamine-1-P
N-Acetyl-Glucosamine-1-P
UDP-N-Acetyl Glucosamine
UDP-N-Acetyl Mannosamin-Uronic Acid
Fructose-6-P
pmi
Mannose-6-P
Mannose-1-P
GDP-Mannose
Mannose
Mannose
Glucose-6-P
Glucose
Glucose-1-P
rha1
TDP-Glucose
rha2
TDP-4-Keto-6-Deoxyglucose
amt
TDP-4-Amino-4,6-Dideoxy-D-Galactose
act
TDP-4-Acetoamido-4,6-Dideoxy-D-Galactose
TDP-Rhamnose
galU
UDP-Glucose
UDP-Galactose
Galactose-1-P
Galactose

Figure 4. ECA Biosynthesis. The synthesis of lipid III is believed to occur on the cytoplasmic face of the inner membrane. The location and mechanisms of polymerization and transfer to phospholipid or LPS are not known and are currently under study. Abbreviations are: GlcNAc, N-acetyl-glucosamine; ManAcA, N-acetyl-mannosaminuronic acid; Fuc4NAc, 4-acetamido-4,6-dideoxy-D-galactose.


UDP–GlcNAc
UMP
UDP–ManAcA
UDP
TDP–Fuc4NAc
TDP
GlcNAc
ManAcA·GlcNAc
Fuc4NAc·ManAcA·GlcNAc
OH
cytoplasmic membrane
Undecaprenyl Monophosphate
Lipid I
Lipid II
Lipid III
Polymerization
Transfer of Polysaccharide to Phospholipid/LPS
Translocation to Outer Membrane

the genes in the *rfb* region. Furthermore, the transfer of the *Salmonella montevideo* or *E. coli rfb* regions into *S. typhimurium* would not support normal levels of ECA synthesis. Indeed, the amount of ECA synthesized in these constructs was reduced to "trace" amounts similar to those *Salmonella* constructs that contained the *S. typhimurium rfe-rff* region alone (Mäkelä and Mayer, 1974; Mäkelä et al., 1976).

Studies to further clarify the role of the *rfb* region involved the use of *rfb* deletion mutants (Mäkelä et al., 1976). Accordingly, strains were constructed that had deletions of various lengths extending from *his* clockwise into the *rfb* region (Nikaido et al., 1967). Through the use of these deletion mutants, two genes involved in the biosynthesis of TDP-rhamnose, *rfbA* which encodes TDPglucose pyrophosphorylase and *rfbB* which encodes TDPglucose oxidoreductase, were implicated in ECA synthesis (Mäkelä et al., 1976; Lew et al., 1986; Nikaido et al., 1966). The role of these enzymes was established when the structure of ECA was determined and shown to contain Fuc4NAc (Lugowski et al., 1983). The synthesis of TDP-Fuc4NAc, the donor of Fuc4NAc residues, utilizes the first two steps of the TDP-rhamnose pathway. Further, the first enzyme, TDPglucose-pyrophosphorylase, which synthesizes TDP-glucose was established as a necessary enzyme in ECA synthesis (Lew et al., 1986).

The Δ*rfb* deletion strains were also shown to produce trace amounts of ECA. Accordingly, it was suggested that small amounts of TDP-glucose were synthesized by UDPglucose pyrophosphorylase which was present and active in these strains (Lew et al., 1986). The "ECA-trace" strains are unstable and eventually accumulate *rfe-rff* mutations rendering them ECA minus. The characterization of these secondary mutations led to the identification of the *rff* genes (Mäkelä et al., 1976). Further studies

determined the function of several gene products from the *rff* region involved in the synthesis of UDP-ManNAcA and TDP-Fuc4NAc (Lew et al., 1978; Lew et al., 1986).

As mentioned previously, the *S. typhimurium* Δ*rfb* deletion strains were observed to have increased sensitivities to many hydrophobic agents, the most prominent of which was sodium dodecylsulfate (SDS). Growth of these mutants in the presence of SDS led to the selection of SDS-resistant suppressor mutations. Seven of the SDS-resistant mutants were fully characterized and determined to have mutations in the *rfe-rff* genes (Mäkelä et al., 1976). It was suggested that the SDS sensitivity of Δ*rfb* mutants was likely due to the accumulation of a lipid-linked intermediate involved in ECA synthesis. However, the possibility that the reduced amount of ECA itself was causing the hypersensitive defect could not be ruled out, since the secondary mutations leading to SDS-resistance eliminated all ECA (Mäkelä et al., 1976). Rick et al., showed that the Δ*rfb* mutants accumulated lipid II (Rick et al., 1988). Lipid II was absent in wild type strains, *rfe* mutants, and in some *rff* mutants. Tunicamycin treatment of the Δ*rfb* mutants abolished the accumulation of lipid II, and rendered the strains ECA-minus. In addition, the SDS sensitivity of these strains was lost when the cells were grown in the presence of tunicamycin. An analysis of a limited number of SDS-resistant derivatives of Δ*rfb* mutants identified lipid I accumulators and strains possessing secondary mutations that precluded accumulation of any intermediates. All of the SDS-resistant derivatives were ECA-minus. The ability of tunicamycin to protect the cells from SDS and the analysis of the SDS-resistant derivatives suggested that the accumulation of lipid II might be the cause of the hydrophobic sensitivity (Rick et al., 1988). However, since *S. typhimurium* mutants that accumulate the ECA

intermediate lipid I do not show increased sensitivity to hydrophobic agents, the synthesis of trace amounts of ECA could not be ruled out as a contributing factor.

The most informative study to date on ECA genetics comes from mapping and biochemical characterization studies of ECA-minus, Tn10-insertion mutants of *E. coli* (Meier-Dieter et al., 1990; Meier-Dieter et al., 1992). These studies precisely mapped and identified the specific functions of many of the individual ECA biosynthetic genes. Genes encoding enzymes involved in the synthesis of the nucleotide sugars, UDP-ManAcA and TDP-Fuc4NAc, as well as ManNAcA and Fuc4NAc transferases and a possible polymerase activity have all been shown to map to the *rff* region (Meier-Dieter et al., 1990).

Lipid I synthesis is abolished in *rfe* mutants (Meier-Dieter et al., 1990). The *rfe* gene is also involved in the biosynthesis of certain O-side chains of *E. coli* and *Salmonella* (Schmidt et al., 1976; Mäkelä et al., 1970). Specifically, the synthesis of *E. coli* O8, O9, O20, and O101 O-antigens and synthesis of the O antigens of *S. minnesota* (type L) and *S. montevideo* (type C1) are all *rfe*-dependent. The genes for the synthesis of the nucleotide-sugars and transferases for these O-antigens are located in their respective *rfb* regions (Mäkelä and Mayer, 1974). The presence of *rfe*-dependent and independent O-antigen synthesis suggested the possibility that the *rfe* region coded for the biosynthesis of an independent lipid carrier molecule. This lipid would function as a carrier of oligosaccharide repeat units for ECA synthesis in all Enterobacteriaceae and for the repeat units of O-side chains in certain species (Mäkelä and Mayer, 1974). However, this seemed to be ruled out when undecaprenyl monophosphate was identified

as the lipid moiety involved in ECA synthesis (Rick et al., 1985) and in the synthesis of O8-, and O9- side chains (Weisgerber and Jann, 1982).

A plasmid containing a single open reading frame cloned from *E. coli* K12 was found to complement several *E. coli rfe* mutants (Meier-Dieter et al., 1992). This open reading frame restored the synthesis of both ECA and O8 antigen in *rfe*::Tn10-insertion mutants. In addition, the presence of this open reading frame on a high-copy plasmid rendered transformants resistant to tunicamycin inhibition of ECA biosynthesis (Meier-Dieter et al., 1992). More specifically, ECA synthesis in wild-type strains of *E. coli* was completely inhibited when tunicamycin was added to cultures to give a final concentration of 2-3ug/ml. In contrast, ECA synthesis in transformants of *E. coli* possessing the open reading frame on a high copy plasmid were not inhibited by up to 20ug/ml of tunicamycin. Accordingly, the open reading frame was identified as the *rfe* gene, and it was concluded that the *rfe* gene codes for the UDP-GlcNAc:undecaprenyl phosphate GlcNAc-1-phosphate transferase, the enzyme responsible for the transfer of GlcNAc-1-phosphate to undecaprenyl phosphate to form Lipid I. However, identification of the function of the *rfe* gene product does not explain the role of *rfe* in O8-side chain synthesis. The O8-side chain and many of the other *rfe*-dependent O-antigens either lack GlcNAc or contain GlcNAc in a position that cannot be explained by a mechanism involving lipid I (Meier-Dieter et al., 1992). Specifically O8 side chains are homopolymers consisting of mannose linked ->3)-α-D-Man-(1-2)-α-D-Man-(1-2)-α-D-Man-(1-> (Reske and Jann, 1972). In addition, previous work indicates that glucose linked to undecaprenol as Glc-pyrophosphorylundecaprenol is the acceptor of mannose residues in O8 and O9 side chain synthesis (Flemming and Jann, 1978). This is, of course,

inconsistent with the structure of lipid I and ECA synthesis. Thus, the role and function of *rfe* in O-antigen synthesis remains unclear.

Very little is known about the biological role of ECA. Only two reports on the possible role of ECA in *S. typhimurium* have been published. A significant difference in the virulence of ECA^+ and ECA^- isogenic strains of *S. typhimurium* in an intraperitoneal mouse model have been reported (Valtonen et al., 1976). However, growth rates and clearance were observed to be the same regardless of the ability of the strains to synthesize ECA. A more recent study has suggested that ECA^+ strains are able to persist longer in mice than ECA^- strains (Nnalue and Stocker, 1987). The ECA^- strains used in this study were actually "trace" strains, and it would be expected that they accumulate lipid II. Given the possible outer membrane defects of these strains, a reduced viability in a biological system is not surprising. A true ECA^- strain was not tested in this study. Therefore, the biological role of ECA in the viability of various Enterobacteriaceae in a host remains unknown.

It has been suggested that the negatively charged ManAcA residues of ECA may play a role in trapping polyvalent cations, particularly Mg^{+2} (Kuhn et al., 1988). This function also remains to be established.

The biosynthesis and presence of ECA in the Enterobacteriaceae presents several puzzles. For example, why does the accumulation of an ECA-lipid intermediate and/or reduced amounts of ECA cause sensitivity to hydrophobic agents in *S. typhimurium*? Similar lipid II accumulating strains of *E. coli* do not produce reduced amounts of ECA nor do they show a hydrophobic agent hypersensivity phenotype. However, lipid II accumulating mutants of *E. coli* also do not make trace amounts of ECA (Meier-Dieter et al., 1992). The role of ECA and ECA biosynthetic intermediates in maintenance and/or disruption of the barrier function of

the outer membrane in *S. typhimurium* may provide insight into outer membrane biogenesis.

The function of the *rfe* gene product in the synthesis of certain O-antigens is still not completely clear. Although it seems likely that the *rfe* gene product indeed functions as a GlcNAc-1-phosphate transferase, the apparent lack of GlcNAc in certain *rfe*-dependent O-antigens is not in agreement with this function. Thus, the available data suggests the possibility that the *rfe* gene is responsible for a step prior to GlcNAc transfer that is common to both ECA synthesis and the synthesis of certain O-side chains. For example, it cannot be ruled out that the *rfe* gene product is a lipid carrier (undecaprenol) modification factor, a compartmentalization factor, or may serve some as yet unrecognized function. Alternatively, it is possible that O8- and O9-side chains possess a single reducing terminal GlcNAc residue that has not been detected in previous structural analyses.

This dissertation attempts to better define the ECA-"trace" phenotype in *S. typhimurium*. Specific emphasis has been placed on determining the biochemical mechanism responsible for the increased sensitivity of mutants possessing the ECA-"trace" phenotype to SDS. This was performed using chemical, biochemical and genetic approaches to define whether lipid II accumulation or the synthesis of trace amounts of ECA are responsible for increased sensitivity of Δ*rfb* mutants of *S. typhimurium* to hydrophobic agents.

Purification and mass spectral analysis of the second ECA intermediate, lipid II were also performed to define the structure and nature of the carrier lipid.

Additional studies were directed toward determining the role of the *rfe* gene and lipid I in the synthesis of O8-side chains in *E. coli* O8. As

previously mentioned, all available information on the mechanism of O8-side chain synthesis fails to provide an explanation for the role of lipid I in this process. In contrast, the data for the *rfe* gene in ECA synthesis strongly suggests that this gene-product is the GlcNAc-1-phosphate transferase. Chemical, biochemical and genetic approaches were used to demonstrate tunicamycin sensitivity and the roles of glucose and N-acetylglucosamine in O8 synthesis.

MATERIALS AND METHODS

Bacterial Strains and Media

Bacterial strains used in these studies are listed in Table 1. Generalized transducing phages P1 and P22 were of the laboratory collection. The generalized transducing phage ES18h-1 was obtained from B. A. D. Stocker's laboratory. Phages C21, Felix-O and Ω8 were used for the phenotypic determination of LPS or O-antigen structure; these phages were of the laboratory collection. All strains were grown with vigorous aeration in either medium A (1.0% proteose peptone, 0.1% beef extract, 0.5% NaCl), medium B (Luria-Bertani medium; 1.0% bacto-tryptone, 0.5% bacto-yeast extract, 1.0% NaCl) or medium C (M9-minimal medium; 1.3% NA_2HPO_4-$7H_2O$, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2mM $MgSO_4$, 0.1mM $CaCl_2$) as indicated (Maniatis et al., 1982). Proteose peptone, beef extract, bacto-yeast extract, and bacto-agar were purchased from Difco Laboratories, Detroit, MI. The addition of carbon sources and other supplements will be specifically mentioned for each experimental procedure. Solid agar and top-agar overlays contained agar at a final concentration of 1.5% and 0.75%, respectively.

Indicator media used in these studies for the evaluation of sugar utilization or for identification purposes include MacConkey agar, triple sugar iron (TSI) agar, tetrazolium (TZ) medium, and brom thymol blue (BTB) medium. TSI agar (Difco) was rehydrated, distributed to tubes and sterilized according to manufacturers instructions. MacConkey agar (Difco) was rehydrated and sterilized according to the manufacturers instructions. TZ

TABLE 1. BACTERIAL STRAINS

STRAIN	RELEVANT PROPERTIES	REFERENCE/SOURCE
Salmonella typhimurium		
LT2	Wild Type	Zinder and Lederberg (1952)
G30	*galE*	Rick and Young (1982a)
PR122	*galE, nag*	Rick and Young (1982a&b)
SH4892	*rfb*4020, *his*5406, *rfe*	Rick et al. (1985)
SH5150	*rff*4270, *ilv*1190, Δ*his*809(*rfb*)	Rick et al. (1988)
SH5187	Δ*his*399(*rfb*)	Mäkelä et al. (1976)
SL3769	*rfa*G471	Roantree et al. (1977)
G30A	*gal*E, Re-LPS	Osborn et al. (1972)
TT10424	F'128 *pro*+, *lac*+, *zzf*1832::Tn10	Way et al. (1984)
TT10427	pNK972(amp,Tn10-transposase)	Way et al. (1984)
SA975	HfrK13(78 minutes cw), *thrA*49, *leuBCD*39	Sanderson et al. (1972)
SL1314	*galU*586(ts), *rpsL*	Subbaiah and Stocker (1964)
SA3858	*rpsL*120, *hsdL*6, *hsdSA*29, *hsdSB*121, *ilv*452, *galE*496, *mutH*101::Tn5	Rayssiguier et al. (1989)
LB5010	*rpsL*120, *hsdL*6, *hsdSA*29, *hsdSB*121, *ilv*452, *galE*496	Bullas and Ryu (1983)
HR104	as SH5150, ilv^{+}, met^{+}	This study
HR111	as SL1314, gal^{+}, tet^{R}	This study
HR112	as SL1314 *galU*586(ts), tet^{R}	This study
HR114	as HR104 *galU*586(ts), tet^{R}	This study

TABLE 1, Continued

STRAIN	RELEVANT PROPERTIES	REFERENCE/SOURCE
HR142	as SA3858 *pgi*::Tn10	This study
HR150	as LT2 *mutH*101::Tn5	This study
HR151	as HR150 *pgi*::Tn10	This study
HR159	as HR104 *pgi*::Tn10	This study
HR197	as SH5150, *ilv*$^+$, *met*$^+$	This study
HR202	as LB5010 with pKO1	This study
HR203	as LB5010 with pPR645	This study
HR206	as SH5150 with pKO1	This study
HR207	as HR197 with pKO1	This study
HR208	as HR197 with pPR645	This study
HR209	as SH5150 with pPR645	This study
HR210	as SA3858 *ilv*$^+$, *zie*2::Tn10, *rff*726	This study
Escherichia coli		
PA200SR	*hisG*, *rpsL*9	Bachmann (1972)
GMS343	*manA*4, *rpsL*700	Novel and Novel (1973)
G146	*bla-zdg*::Tn10	Grogan and Cronan (1984)
2442	O8$^-$	Meier-Dieter et al. (1989)
2443	as 2442 O8$^+$	Meier-Dieter et al. (1989)
P72	Hfr (102 minutes cw)	Jacob and Wollman (1961)
SV107	*pgi*::Tn10	Rowely and Wolf (1991)

TABLE 1, Continued

STRAIN	RELEVANT PROPERTIES	REFERENCE/SOURCE
21731	*rff*726, *zie*2::Tn10	Meier-Dieter et al. (1990)
P3778	pPR645	Jiang et al (1991)
N100	pKO1	McKenney et al. (1981)
PR4000	as GMS343 with *bla-zdg*::Tn10	This study
PR4024	as PA200SR with *bla-zdg*::Tn10 , *manA* 4	This study
HR188	as PR4024 tet^S	This study
HR191	as HR188 with *pgi*::Tn10	This study
HR193	as HR191 his^+, $O8^+$	This study

medium contained (per liter) 25.5gm Bacto-antibiotic medium 2 (Difco), 5ml of a 1% solution of 2,3,5 triphenyl-2-H-tetrazolium chloride, and 15gm agar (Davis et al., 1980). BTB medium contained 7.5gm yeast extract, 200mg brom thymol blue and 15gm agar per liter of medium C (Subbaiah and Stocker, 1964). For MacConkey, TZ, and BTB media, the appropriate sterile sugar solution was added after autoclaving to a final concentration of 1% (MacConkey and TZ) or 0.5% (BTB).

RNA containing plates were used to test for leakiness of periplasmic enzymes. These plates contained 20ml of medium A-agar with a 4ml overlay of 1% yeast RNA (Sigma) in medium A-agar (Weigand and Rothfield, 1976). Strains were transferred to plates and after overnight growth at 37°C, the plates were flooded with 0.5N HCl for 3 minutes. Strains leaking periplasmic RNase showed large halos around individual colonies.

Bacterial Strain Construction Techniques.

Conjugation procedures were modifications of previously described methods (Sanderson et al., 1972; Rayssiguier et al., 1989). In method-I, both Hfr-donor and recipient strains were grown separately overnight in 10ml medium A at 37°C. Cultures were then centrifuged (9,000xg) and washed in sterile 0.9% saline. The washed cells were then resuspended in 1.0ml of sterile 0.9% saline. For mating, 0.1ml of the recipient strain was spread on selective media and allowed to dry. The Hfr-donor strain was then placed on the recipient lawn in 5ul amounts, and the plates were incubated overnight at 37°C.

Method-II was employed for interrupted matings. Hfr and recipient strains were grown to mid-log phase in medium B at 37°C after transfer

from overnight cultures. Cultures were adjusted to an OD_{600nm} of 0.5. Equal volumes of the cultures were then mixed and 0.1ml of the mixture was immediately deposited on a sterile 0.45μ filter. Prior to mixing the cultures, the filters were placed on plates containing medium B and pre-incubated at 37°C. After addition of the mating-mixture, the plates were again incubated at 37°C. The filters were then removed from the plates at various intervals and washed three times with 0.1ml of medium B. The washes were pooled, mixed vigorously, and then plated on selective media and incubated overnight at 37°C.

Phage lysates for transductions were made by mixing 0.1ml of serial dilutions of phage with 0.1 ml of log-phase donor cells grown in medium B plus 0.2% glucose. The phage and cells were mixed in 2.5ml top-agar (medium B) and then poured over medium B plates and incubated overnight at 37°C. Plates showing confluent lysis were flooded with 3.0ml of medium B and incubated at 4°C for four to six hours. The liquid from the plates was then pooled and a small amount of chloroform (100ul/ml) was added with mixing. The lysates were then titered on the recipient strain. For phage P1, all media were supplemented with 5mM $CaCl_2$ and 10mM $MgSO_4$.

Phage P1 was used for all *E. coli* constructions and for some *E. coli* to *S. typhimurium* transductions where the recipient strain possessed a Rc-chemotype LPS. The recipient strain was grown to mid-log phase in medium B supplemented with 0.2% glucose, 5mM $CaCl_2$, and 10mM $MgSO_4$. The culture was then centrifuged (9000xg) and resuspended in 5mM $CaCl_2$/10mM $MgSO_4$. Phage and cells were mixed to give multiplicities of infection (MOI) ranging from 10:1 to 1:1. Mixtures were incubated at 30°C for 30 minutes to allow phage adsorption and adsorption was then terminated by the addition of 100ul of 1.0M citrate. Medium B was next

added and the mixtures were again incubated 30°C for 1 hour to allow for phenotypic expression in cases involving transduction of an antibiotic resistance determinant. The mixture was then plated on selective media and incubated at 37°C.

Phage P22 was used for *S. typhimurium* constructions. The P22 transduction procedure employed was similar to that used for P1 transductions except that an overnight culture of the recipient strain was used and calcium/magnesium supplements were not required. The phage adsorption step was terminated with 10mM ethylene glycol bis(ß-aminoethyl ether)-N,N'-tetracetic acid (EGTA). The selection plates therefore also contain 10mM EGTA.

Phage ES18h-1 was also used to transduce *S. typhimurium*. The phage/cell mixture was incubated for 45 minutes at 30°C and then centrifuged and washed one time with 0.9% saline in order to terminate transduction. The cells were then resuspended in 0.2ml of medium B or medium C and incubated at 30°C for one hour before spreading on selective plates.

Methods for the isolation of plasmids and bacterial transformation were the same as those described by Sambrook et al., (Sambrook et al., 1989). Plasmid DNA was isolated from cultures grown overnight in medium B using the alkali lysis method. The size of plasmids and restriction fragments were determined by agarose gel electrophoresis using 1% gels. Plasmid restriction endonuclease digestions were performed according to manufacturer's instructions. For competent cells, strains were grown to mid-log phase (OD_{600nm} of 0.4) in 15ml of medium B with 0.5% glucose. The cells were harvested at 9,000xg for ten minutes, resuspended in 10ml of 10mM $MgSO_4$ and incubated on ice for 30 minutes. The bacteria were again

harvested, resuspended in 5ml of 50<u>mM</u> $CaCl_2$ and incubated on ice for 15 minutes. The bacteria were harvested and resuspended in 0.5ml of cold 50<u>mM</u> $CaCl_2$ and transformed by mixing 200ul with 5ul of isolated plasmid. Bacteria were heat-shocked for 2.5 minutes at 42^{o}C and then incubated at room temperature for 15 minutes. Medium B was added (0.5ml) and incubation was continued at 37^{o}C fro 1.5 hours. The transformation mixture was then spread over medium B plates containing 100ug/ml ampicillin and incubated at 37^{o}C. Transformants were picked and restreaked for isolation on medium B/ampicillin plates.

For some transformations, cells were prepared for electroporation using modifications previously described (Ostrovsky De Spicer et al,. 1991). Briefly, cells were grown overnight at 37^{o}C in medium B, and 1.0ml of the overnight culture was used to inoculate 50ml of medium B. The resulting culture was incubated with shaking at 37^{o}C for 90 minutes, and the cells were then chilled on ice for 15 minutes prior to harvesting by centrifugation at 10,000xg for 10 minutes at 4^{o}C. The bacteria were then washed sequentially with 25ml and 10ml of cold 1<u>mM</u> N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 5.2. The washed cells were finally washed with 10ml cold 272<u>mM</u> sucrose/15% glycerol and resuspended in 1ml of the same solution for electroporation. Forty microliters of bacteria and 1-5ul of plasmid were mixed immediately in a cold 0.2cm electroporation cuvette prior to pulsing. Electroporation was performed using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, Calif). Instrument settings were: voltage, 1.25(+) kV; capacitance, 25uF; and resistance, 800-1000 ohms. Mixtures were pulsed once and time constants ranged from 18.5 to 21.9msec. The pulsed mixtures were immediately transferred to 1ml of prewarmed medium B and incubated for 90 minutes at

37°C. Transformants were selected by transferring 200ul/plate to medium B plates containing 100ug/ml ampicillin and incubating at 37°C.

A random Tn10 insertion pool in *S. typhimurium* was created by P22-mediated transduction of a transposase defective Tn10 from *S. typhimurium* TT10424 into a transposase containing strain, TT10427 (Amp^R). Both the defective Tn10 and the transposase genes were located on separate plasmids. TT10427 was grown overnight in medium B containing 100ug/ml ampicillin. One-milliliter of the overnight culture was transferred to 30ml of medium B containing 100ug/ml ampicillin. Cells were grown to an OD_{600nm} of 1.0, and then centrifuged at 5,000xg for ten minutes. The pelleted cells were resuspended in medium B with 5mM $CaCl_2$ to a cell density of $5x10^9$ cell/ml (OD_{600nm} of $1.0=10^9$ cell/ml). The P22 lysate of strain TT10424 was added to strain TT10427 to give an MOI of 0.8, mixed, and incubated at 37°C for thirty minutes. The mixture (100ul) was then spread over each of 20 medium B plates containing 10mM EGTA and 25ug/ml tetracycline; the plates were then incubated overnight at 37°C. Transductants were harvested by the addition of 1.0ml of medium B containing 10mM EGTA to each plate and the colonies were aseptically mixed into a suspension. The suspensions from each plate were pooled and transferred to 200ml of medium B containing 10mM EGTA, 0.2% glucose, and 25ug/ml tetracycline and then incubated for one hour at 37°C. The bacteria were harvested and washed two times in medium B by centrifugation at 9,000xg for ten minutes at room temperature. The washed cells were resuspended in medium B/0.2% glucose to a density of $2x10^9$ cells/ml, mixed with P22 to a MOI of 0.01, and incubated overnight at 37°C. Chloroform (2ml/50ml of culture) was then added with mixing to the overnight culture, and the culture was centrifuged at 10,000xg for ten minutes. The supernatant solution was transferred to a

sterile bottle followed by the addition of 1-2ml chloroform. This P22 lysate was then titered on a *S. typhimurium* strain with smooth LPS as previously described.

Strains were cured of tetracycline resistance (due to the transposon Tn10) by the fusaric acid selection method (Maloy and Nunn, 1981). Bacterial strains were grown to an OD_{600nm} of 0.6 in medium B and then twice serially diluted 1:10 in medium C. Undiluted and diluted cells (0.1ml) were plated in triplicate on fusaric acid containing plates and incubated at 37°C. Colonies were picked and streaked for isolation on medium B plates. Isolated colonies were then screened for tetracycline sensitivity and other phenotypic properties as appropriate. The composition of the fusaric acid containing plates (per liter) was as follows: 15gm agar, 5gm tryptone, 5gm yeast extract, 10gm NaCl and 4ml of a 12.5mg/ml solution of chlortetracycline. After sterilization the solution was allowed to cool to 47°C and 20ml NaH_2PO_4(0.5gm/ml), 6ml fusaric acid(2mg/ml) and 5ml $ZnCl_2$ (20mM) were added (Maloy and Nunn, 1981). Plates were then poured.

Strains were routinely tested for SDS-sensitivity by streaking the strain across a medium A plate so as to produce confluent growth and then placing a sterile 1/4 inch disk (Difco) saturated with a sterile 1% SDS solution on the surface of the plate in a heavily inoculated region. Plates were incubated overnight at 37°C, and then scored for growth inhibition. An inhibition zone greater then 1.5cm in diameter indicated SDS-sensitivity.

The sensitivity of strains to phage was determined by the cross-streak method. A single streak of a phage lysate was made down the center of a medium B plate. The streak was allowed to dry, and strains to be tested were cross-streaked at right angles over the phage lysate line using a "fine" inoculating needle. Plates were incubated 37°C overnight and phage

sensitivity was indicated by interruption of the growth streak at and beyond the lysate line. Strains with known sensitivity patterns were included on the plates as controls.

All bacterial strains were stored frozen at -80°C after washing cells grown overnight in medium B-0.2% glucose with 0.9% NaCl and then resuspending the washed cells in 15% glycerol/0.85% NaCl.

Colony Blots

Bacterial strains were screened for ECA production using the colony immunoblot procedure of Meier-Dieter et al. (Meier-Dieter et al., 1989). Single colonies were transferred to medium B plates and incubated at 37°C overnight. A nitrocellulose filter was placed over the colonies and incubation at 37°C was continued for one hour. The filter was then removed and baked at 80°C for four hours. The filter was next washed four times with TN-buffer (50mM Tris-HCl, pH7.5, 0.9% NaCl); each washing was carried out for 15 minutes. After washing, the filters were placed in TN-buffer containing 3% bovine serum albumin (BSA) and mixed gently for one hour. The filter was then gently mixed for two hours with mouse anti-ECA monoclonal antibody MAb898 (Peters et al., 1985) diluted 1:500 in TN-buffer containing 3% BSA. The filter was subsequently washed four times with TN-buffer and then gently mixed for two hours with goat anti-mouse IgG-peroxidase conjugated antibody which was diluted 1:1000 in TN-buffer containing 3% BSA. The filter was then washed four times with TN-buffer. ECA-bands were detected by gently mixing the filter in a peroxidase substrate reaction mixture consisting of 30mg 4-chloro-1-naphthol in 10ml of methanol mixed with

50ml of TN-buffer and 20ul of 30% H_2O_2. After development, the filters were washed in water and dried at room temperature.

Membrane Separation

Membrane fractions were isolated, with modifications, according to the method of Osborn and co-workers (Osborn et al., 1972, Osborn and Munson, 1974). Cells were harvested by centrifugation at 10,000xg for ten minutes and resuspended to seven times the culture's final OD_{600nm} in 0.75M sucrose, 10mM Tris-HCl buffer, pH 7.8. This and all subsequent steps were performed at 4°C. Spheroplasts were prepared by addition to the cell suspension of a 2mg/ml lysozyme solution to give a final concentration of 100ug lysozyme/ml; the suspension was then incubated on ice for two minutes. Two times the suspension volume of 1.5mM EDTA was then added slowly over a ten minute period while gently swirling the mixture. The resulting spheroplasts were then lysed by sonication. The lysates were centrifuged at 1,200xg for twenty minutes to remove unbroken cells. The supernatant was then centrifuged at 360,000xg for two hours to harvest the membranes. The membrane pellet was resuspended in 10ml of 0.25M sucrose, 3.3mM Tris-HCl pH7.8, and 1.0mM EDTA by mild sonication. The resuspended membranes were centrifuged at 360,000xg for two hours and then resuspended by mild sonication in 0.5ml of 25% sucrose, 5mM EDTA. This suspension was layered over a 30-50% sucrose step gradient (see below) and centrifuged 35,000RPM for twenty hours at 4°C in an Beckman-SW40 swinging bucket rotor. Following centrifugation, the gradients were fractionated into 0.25ml fractions by removal of the fractions from the top of the tube. In all cases the cells were labelled by the addition of either

[2-^{3}H]glycerol or L-[^{35}S]methionine during growth to label phospholipids or protein, respectively, in order to facilitate detection of membrane-containing fractions. Fractions containing inner or outer membranes were pooled, diluted to 10ml with 50mM Tris-HCl, pH7.5, and centrifuged at 360,000xg at 4°C for two hours. Membrane pellets were then frozen and stored -20°C. For preparations in which the activity of the cytoplasmic membrane marker enzyme, NADH oxidase, was to be determined, dithiothreitol was added to all solutions to give a final concentration of 0.2mM . The NADH oxidase activity of membrane fractions was determined prior to storage.

Sucrose solutions were prepared as follows (weight to weight): 69.2g (55%), 61.5g (50%), 54.1g (45%), 47.1g (40%), 40.3g (35%), 33.8g (30%), 27.6g (25%). Agiven amount of sucrose was dissolved in a minimal volume of H_2O to which was added 5ml of a 100mM EDTA, pH 7.5, stock solution; the solution was then brought to a final volume of 100ml by the addition of H_2O to yield the sucrose solution w/w% indicated in parenthesis. Gradients were made by layering 2.1 ml of the 50% through 30% solutions over 0.5ml of the 55% sucrose solution in a 13.5ml tube (Osborn and Munson, 1974).

Cell Envelope "P160" for In Vitro Polymer Synthesis

Experiments designed to look at the in vitro synthesis of O8 side-chain synthesis required isolated bacterial cell envelopes. The cell envelope preparation was prepared as follows: bacteria were grown in 200ml of medium A containing 0.2% glucose to an OD_{600nm} of 0.6. The bacteria were harvested and washed once with 0.9% saline at 9,000xg for 10 minutes. The cells were resuspended in 1.0ml 15mM Tris-HCl, pH 8.0,/2mM 2-mercaptoethanol (2-ME) and then disrupted by sonication. The sonicated

mixture was then centrifuged at 160,000xg for one hour at 4°C, and the pellet was resuspended in 0.3ml of 15mM Tris-HCl, pH 8.0 (P160) (Barr et al., 1988).

Minimum Inhibitory Concentration (MIC)

The determination of the susceptibilities of the various bacterial strains to antibiotics and hydrophobic agents was measured by standard methods using previously described microdilution test procedures (Washington and Sutter, 1980; Gavan and Barry, 1980).

Primary stock solutions of antibiotics and hydrophobic agents were made in distilled water (unless noted) as follows: ampicillin, 10mg/ml; pennicillin G, 1mg/ml; tetracycline, 25mg/ml(in methanol); oxytetracycline, 2mg/ml; carbenicillin, 1mg/ml; cephalosporin C, 1mg/ml; actinomycin D, 1mg/ml; kanamycin, 50mg/ml; chlortetracycline, 1mg/ml; vancomycin, 100mg/ml; malachite green, 2mg/ml; erythromycin, 2mg/ml; bacitracin, 20mg/ml; streptomycin, 50mg/ml; crystal violet, 100mg/ml; novobiocin, 5mg/ml; rifamycin, 1mg/ml(in methanol); cloxacillin, 2mg/ml; dicloxacillin, 5mg/ml; polymyxin B, 1mg/ml; SDS, 100mg/ml; sodium cholate, 300mg/ml; sodium deoxycholate, 30mg/ml; Triton X100, 100mg/ml; and methylbenzylethonium chloride (MBE), 100mg/ml. Secondary stock solutions were then prepared by diluting each of the primary stock solutions to give twice the required highest-concentration in the medium used for the microdilution test. These concentrations were based on reported literature values (Sanderson et al., 1974; Nikaido, 1976; Roantree et al., 1977; Sukupolvi et al., 1984; Barry and Thornsberry, 1980) and some trial experiments to determine inhibitory ranges.

One-hundred fifty microliters of the test media was added to all wells of a sterile 96-well microtiter plate. Serial two-fold dilutions of each agent were then made by adding 150ul of the secondary stock solutions to the first well of each row, mixing, and then serially transferring 150ul to all subsequent wells except the last well. The 150ul from the next to the last well was discarded after mixing. In all cases the last well served as a media control. The initial concentrations of each agent in the first well of each row were as follows: ampicillin, 9.4ug/ml; pennicillin G, 375ug/ml; tetracycline, 7.5ug/ml; oxytetracycline, 5.6ug/ml; carbenicillin, 375ug/ml; cephalosporin C, 15ug/ml; actinomycin D, 375ug/ml; kanamycin, 5.6ug/ml; chlorotetracycline, 3.8ug/ml; vancomycin, 900ug/ml; malachite green, 60ug/ml; erythromycin, 75ug/ml; bacitracin, 2.3mg/ml; streptomycin, 525ug/ml; crystal violet, 38ug/ml; novobiocin, 75ug/ml; rifamycin, 150ug/ml; cloxacillin, 263ug/ml; dicloxacillin, 375ug/ml; polymyxin B, 1.5ug/ml; SDS, 750ug/ml; sodium cholate, 45mg/ml; sodium deoxycholate, 900ug/ml; triton X100, 38ug/ml; and methylbenzylethonium chloride(MBE), 370ug/ml.

Bacterial strains were grown overnight in the test medium and then diluted to $2x10^5$ bacterial/ml in fresh media(0.1 $OD_{600nm} = 10^8$ bacteria/ml). The diluted strains were then inoculated by adding 50ul (10^4 bacteria) to all wells. The microtiter plates were subsequently incubated at 37^oC for 24 hours. The MIC was defined as the lowest concentration which prevented detectable growth (Gavan and Barry, 1980).

Bacterial Cell Lysis Assays

In order to determine the rates of bacterial cell lysis by SDS or lysozyme, strains were grown to mid-log phase in medium A supplemented with 0.2% glucose. Various concentrations of SDS or various combinations of lysozyme and EDTA were then added to aliquots of the cultures in cuvettes and the OD_{600nm} was monitored. The initial rates (0-30 seconds) were used to determine lysis rates. The rates of spheroplast lysis by SDS were also determined by this procedure.

Harvesting Large Scale Bacterial Cultures

Bacteria from large scale (50-100 liters) cultures were harvested and concentrated to 4 liters with a Millipore Pellicon cross-flow filtration system. Cells were washed 3 times with 4 liters of 0.9% cold (4°C) saline and further concentrated to a volume of 2 liters. Cells were then harvested by centrifugation at 10,000xg for 10 minutes at 4°C.

LPS Isolation

Lipopolysaccharide (LPS) was isolated according to the procedure of Galanos (Galanos et al., 1969). Cells were grown at 37°C with vigorous aeration in 50 liters of medium A containing 0.2% glucose to an OD_{600nm} of 1.0 in a 150 liter Fermatron fermentor (New Brunswick) and subsequently harvested by cross-flow filtration (Millipore). The cells were then resuspended and washed one time each with 200ml of H_2O, 200ml of 95% ethanol, and 200ml of acetone. The acetone washed cells were finally washed

twice with 200ml of diethyl-ether. After each wash the particulate fraction was harvested by centrifugation at 10,000xg for 10 minutes. The ether-washed cells were then dried under vacuum. The dried pellet was next extracted with phenol(90gm in 11ml of water)/chloroform/petroleum ether (PCPE), 2:5:8 (v/v/v), using 4ml of this solution per gram of dried pellet. Extractions were performed by homogenization for two minutes at 0-4°C. The resulting suspension was centrifuged at 10,000xg for 15 minutes at 4°C and the supernatant solution was filtered through Whatman #1 filter paper. The pellet was reextracted with an equal volume of PCPE in the same manner, and the combined extracts were dried under reduced pressure to remove the chloroform and petroleum ether. The phenol-residue was transferred to a centrifuge bottle, and water was added dropwise until the LPS precipitated (0.4ml water/20ml original solvent). The precipitate was isolated by centrifugation at 5,000xg for 10 minutes at room temperature, and the supernate was carefully decanted. The precipitate was washed with 2-3 small volumes of 80% phenol followed by three washes with diethyl-ether. The washed precipitate was then dried under vacuum. The residue was resuspended in water (1ml/gm original dried pellet weight), gently warmed to 45°C and again placed under vacuum to remove any air. The solution was mixed well and centrifuged at 100,000xg at 4°C for four hours. The supernatant solution was discarded and the pellet was resuspended in water, and freeze dried. The final residue was sealed and stored at -20°C.

For analysis of the oligosaccharide region, 5mg of the isolated LPS preparation was resuspended in 0.5ml of 0.1<u>N</u> acetic acid in a 1.5ml eppendorf tube and heated in a 100°C water bath for one hour. Purified LPS chemotype standards (List Biological Laboratories, Inc) were treated in an identical manner. The suspensions were cooled and centrifuged (setting 9

Beckman Table-Top Centrifuge) for 30 minutes. The supernates were transferred to new tubes and reduced to dryness under vacuum at room temperature. For analyses, the residue was dissolved in 1.0ml of water and further diluted 1:10 in water.

Analytical Techniques

Western blot analysis was routinely used to detect the presence of ECA after SDS-gel electrophoresis. The procedure used was that described previously (Rick et al., 1985). Strains were grown overnight in medium A, unless specified. Cells were centrifuged 9,000Xg for 10 minutes at 4°C and washed two times in 0.9% saline. The OD_{600nm} of the resuspended cells was then adjusted to 0.8 with saline. Two-milliliters of the culture was centrifuged and the pelleted cells were resuspended in 100ul of sample buffer (62.5mM Tris-HCl, pH6.5; 5% 2-mercaptoethanol; 2% SDS; 12% glycerol). The suspension was boiled at 100°C for 5 minutes and then subjected to centrifugation to remove insoluble material. The supernatant was transferred to a clean tube and 1ul of 1% bromo-phenol blue was added.

Samples were applied to a 1.5mm thick, 12% polyacrylamide (PA) gel containing SDS and electrophoresed at 25mA for three hours as previously described (Laemmli, 1970). The composition of the running buffer was 3.1gm Tris-HCl, 14.4gm glycine, and 1.0gm SDS in one liter of water. The pH of the running buffer was 8.4. Transfer from the 12% PA-gel to nitrocellulose paper was performed electrophoretically in 25mM Tris-HCl/192mM glycine, pH 8.3, in 20% methanol at 25V for two hours followed by 50V for two hours. The transfer apparatus was water cooled during the transfer. After transfer, the nitrocellulose was placed in buffer A (10mM K_2HPO_4/KH_2PO_4,

pH 7.5, 15mM NaCl) containing 2% gelatin and mixed gently overnight at 4°C. The nitrocellulose was washed three times for 15 minutes with buffer A. The nitrocellulose was then gently mixed for two hours with mouse anti-ECA monoclonal antibody MAb898 (Peters et al., 1985) diluted 1:200 in buffer A/2% gelatin. The nitrocellulose was next washed three times with buffer A, and then gently mixed for two hours with goat anti-mouse IgG-peroxidase conjugate diluted 1:1000 in buffer A/2% gelatin. The nitrocellulose was subsequently washed three times with buffer A, and ECA-bands were detected by gently mixing the nitrocellulose sheet in a peroxidase substrate reaction mixture. The peroxidase reaction mixture was 10mg 3,3'-diaminobenzidine-4HCl in 80ml of 20mM Tris-HCl/0.5M NaCl, pH 7.5, and 4ul of 30% H_2O_2. After development, the blot was washed in water and dried at room temperature.

Radioactively labeled ECA was detected on SDS-gels by fluorography. Strains were grown in medium A and labeled with [^{3}H]GlcNAc (see below) and samples were prepared and analyzed by SDS-gel electrophoresis as described above. After electrophoresis was complete, the gel was transferred to a solution of 45% methanol, and 10% acetic acid in water and then gently mixed for one hour. The gel was next incubated in a solution of En^{3}Hance (Dupont,NEN) for one hour and then transferred to a solution of cold 1% glycerol (4°C) in which it was gently incubated for an additional thirty minutes. The gel was then placed on 3mm chromatography paper (Whatman) and dried at 60°C under reduced pressure for one hour. The dried gel was transferred to a film-casette and exposed to XAR-5 X-ray film (Kodak) at -80°C for an appropriate period of time (Rick et al., 1983).

The in vivo accumulation of lipid-linked ECA intermediates was determined as previously described (Rick et al., 1988). Strains were grown in

200ml of medium A supplemented with 0.2% glucose at 37°C. At mid-log phase (OD_{600nm} of 0.6), the cells were harvested by centrifugation at 9,000xg for 10 minutes at room temperature and resuspended in 25 ml of fresh medium A. The cells were then incubated with 150uCi of [1-^{3}H]GlcNAc (11.2 Ci/mmole) for an additional 30 minutes at 37°C. The labeled cells were harvested by centrifugation at 9,000xg for 10 minutes and washed with 0.9% saline. The bacterial pellet was then washed once with 95% ethanol and once with cold acetone. The pellet was then dried under vacuum. The resulting acetone powder was then extracted with chloroform/methanol (3:2, v/v), and the extract was washed with 20% of the volume of 4mM $MgCl_2$ in a conical tube. The lower organic phase was washed a second time with the upper phase of a mixture of four parts chloroform/methanol (3:2, v/v) and one part 4mM $MgCl_2$. The lower organic phase was removed and evaporated to dryness under a stream of nitrogen. The residue was either resuspended in chloroform/methanol/water (10:10:3, v/v/v) for analysis or stored at -20°C under nitrogen until use.

For in vitro assays requiring lipid I as a substrate, a residue as described above for ECA intermediates was prepared from strain SH5150 resuspended in 1.0ml of chloroform/methanol/water (10:10:3, v/v/v) and transferred to a 1x8cm column of DEAE cellulose (acetate form). The column was equilibrated with chloroform/methanol/water (2:3:1, v/v/v). After loading, the column was washed with 25ml of methanol and 25ml of chloroform/methanol/water (2:3:1) and eluted with a linear gradient of 15mM ammonium acetate in chloroform/methanol/water (2:3:1). The peak fractions were pooled and the solvent was removed under reduced pressure. The residue was resuspended in 1.0ml of chloroform/methanol (3:2, v/v) and washed two times with 0.5ml of water.

Paper chromatography of lipid-linked ECA intermediates was performed on silca-gel-impregnated (SG81, Whatman) papers. SG81 papers were pretreated with 0.068M EDTA, pH 7.7, and allowed to dry at room temperature. Thin layer chromatography of lipid-linked ECA intermediates was performed using silica gel, LK5-TLC plates(Whatman). SG81 papers and TLC plates were activated at 110°C for ten minutes prior to use.

Solvent systems used for chromatography were solvent A, chloroform/methanol/water (65:25:4, v/v/v); solvent B, chloroform/methanol/water/15.1N ammonium hydroxide (88:48:10:1, v/v/v/v); and solvent C, chloroform/methanol/water (10:10:3, v/v/v).

The location of radioactive compounds on thin layer plates was determined by scraping 1cm sections from lanes and placing the silica in Ready-Safe scintillation cocktail for counting. The location of radioactive compounds on SG81 papers was determined by cutting 2cm wide lanes into 1cm sections and soaking each section in 0.25ml of 1.25%SDS for 12 hours at 42°C, prior to the addition of cocktail for counting.

For visualization of lipid intermediates on thin layer plates, the plates were air-dried after development and then sprayed with cupric acetate reagent. The sprayed plates were "ashed" at 210°C for 5 minutes to allow visualization of intermediates. The cupric acetate reagent contained 3gm cupric acetate and 10ml 85% phosphoric acid in 100ml of water.

The carbohydrate portion of ECA-lipid intermediates was identified by P2 gel-permeation chromatography. The carbohydrate was released by treating dried preparations of isolated lipid-intermediates with 0.2ml of 0.1N HCl at 100°C for 15 minutes. The hydrolysates were cooled, dried in vacuo over NaOH pellets and P_2O_5, and dissolved in 0.2ml of water. The total radioactivity in preparations was determined prior to chromatography. Gel -

permeation chromatography was performed using Bio-Gel P2 (Bio-Rad Laboratories, Richmond, Calif.). A 1.5 X 90cm column of P2 was washed and equilibrated in 25mM acetic acid/1mM EDTA. Samples (10,000 dpm in 30ul) were applied to the column along with 10ul each of 100mM solutions of GlcNAc, chitobiose ($GlcNAc_2$), and chitotriose ($GlcNAc_3$) standards. The column was eluted with the same solvent used for equilibration, and 1ml. fractions were collected (Barr et al., 1989). One-half milliliter of each fraction was counted and 0.2ml of each fraction was assayed for reducing sugars by the ferricyanide procedure (see below).

DEAE-cellulose (Whatman DE-52) was prepared by suspending the matrix in 0.5M NaCl followed by washing with the same solution. The DEAE was then converted to the acetate form by washing with concentrated acetic acid until no chloride was detected. The DEAE was next washed with several volumes of methanol and then poured. The poured columns were washed and equilibrated with the appropriate starting solvent system.

Sodium Borohydride Reduction, Total Hydrolysis and N-Acetylation.

One milligram of the carbohydrate sample was resuspended in 0.5ml of 1M $NaBH_4$ and incubated at 4°C for 36 hours (Rick et al., 1985). The reaction was terminated by the slow addition of 4N HCl to neutralize any remaining borohydride. The solution was reduced to dryness under vacuum and resuspended in 0.5ml of acidic methanol (10ul acetic acid in 2ml methanol) repeatedly (4x). The final residue was resuspended in 250ul of water and loaded onto a 1x32cm Bio-Gel P2 column (equilibrated with water). The column was eluted with water and 0.5ml fractions were

collected. Fractions containing the reduced material were pooled and freeze dried.

Total acid hydrolysis of samples was carried out by resuspending the samples in 250ul of 1N HCl and incubating them at 100°C for 4 hours in vacuo. After cooling, the solutions were frozen and reduced to dryness under vacuum.

N-Acetylation was achieved by resuspending residues in 1.0ml of a saturated $NaHCO_3$ solution followed by the addition of 1.0ml of freshly prepared 5% acetic anhydride (0-4°C). Reactions were allowed to proceed at room temperature for one hour, stopped by the addition of 125ul of 4N HCl, followed by further incubation for 15 minutes. The samples were then neutralized by the addition of 150ul of 4N NaOH (Rick et al., 1977). The samples were then freeze dried.

Carbohydrate Analyses

Carbohydrate analyses were performed using a high-performance anion-exchange chromatography system with a pulsed amperometric detector, (HPAE-PAD, Dionex BioLC, Sunnyvale,CA) (Hardy and Townsend, 1988). The instrument was equipped with an Eluant Degas module and all eluants were sparged and pressurized with helium. Carbohydrates were separated on a Dionex CarboPac PA-1 anion exchange resin column (4.6x250mm) fitted with a CarboPac PA guard column. Detection was by PAD using a gold working electrode with the following pulse potentials and durations: E1= 0.05/5, E2= 0.6/2, E3= -0.6/1; the response time was set to three seconds. Data was collected, processed, and plotted using Dionex AI450 software.

Assays

Protein Assay- Protein concentrations were determined by the BCA Protein Assay reagent (Pierce Chemicals) using the "standard protocol" at 37°C for thirty minutes. The chromophore was detected at OD_{562nm}; bovine serum albumin (BSA) was used as a standard.

Total Phosphate Assay- The determination of inorganic and organic phosphate in samples was performed as previously described (Ames, 1966). Total phosphate in organic samples was determined by mixing 10-50ul of the sample with 30ul of 10% $Mg(NO_3)_2 \bullet 6H_2O$ in 95% ethanol and ashing the mixture under a flame. The residue was resuspended in 300ul of 0.5N HCl, heated at 100°C in a water bath for 15 minutes and then cooled to room temperature. To each of the cooled samples was added 700ul of reagent containing one part of a fresh 10% ascorbic acid solution mixed with six parts of a 0.42% ammonium molybdate$\bullet 4H_2O$ solution. The samples were incubated at 45°C for 30 minutes, cooled, and the OD_{820nm} was then determined. Inorganic phosphate was determined by assaying samples without ashing. Phosphate concentrations were determined from a standard curve by assaying a range of known K_2HPO_4 concentrations. Organic phosphate was determined by subtracting inorganic phosphate from total phosphate for each sample. The stock 0.42% ammonium molybdate$\bullet 4H_2O$ reagent was made by mixing 28.6ml of H_2SO_4 and 4.2gms of ammonium molybdate$\bullet 4H_2O$ in a total volume of one liter with water.

NADH Oxidase Assay- NADH oxidase activities were determined in membrane fractions by measuring the decrease in OD_{340nm} in reaction

mixtures containing 5mM Tris-HCl, pH 7.5, 0.12mM NADH, 0.2mM dithiothreitol and 50-100ul of membrane fractions (10-15ug protein) in a total volume of 1ml. Reactions were also measured in the presence of 16mM NaCN in order to determine background NADH oxidation. An extinction coefficient of 6.22cm^{-1}umole was used to calculate enzyme activity (Osborn et al., 1972).

KDO Assay- The KDO content of membrane fractions was determined by precipitating 0.2mg protein equivalent of the fractions with 5.0ml of 10% TCA. The precipitate was centrifuged at 20,000xg for ten minutes and washed two times with water. The precipitate was then resuspended in 0.7ml of 0.018N H_2SO_4 and hydrolyzed by heating at 100°C for 20 minutes in a water bath (Osborn et al., 1972). After cooling, the sample was mixed with 0.7ml of periodate reagent (0.025N HIO_4 and 0.125N H_2SO_4) and incubated for 20 minutes at room temperature. The samples were then mixed with 1.4ml of 2% $NaAsO_2$ in 0.5N HCl and incubated for an additional two minutes at room temperature. Finally, 6.6ml of a fresh solution of 0.3% thiobarbituric acid was added, and the mixture was heated at 100°C for two minutes in a water bath. The solution was cooled and the at OD_{549nm} was determined (Ashwell, 1966). The concentration of KDO was then determined based on an extinction coefficient of 6.41 $cm^{-1}umole^{-1}$.

Ferricyanide-Reducing Sugar Assay- GlcNAc, chitobiose, and chitotriose standards used for P2 gel-permeation chromatography were detected in column fractions by the ferricyanide method (Park and Johnson, 1979). The reagents were; reagent I, 1.325gm Na_2CO_3, 162.5mg KCN in 250ml water; reagent II, 125mg $K_3Fe(CN)_6$ in 250ml water; and reagent III, 2.7gm

$FeNH_4(SO_4)_2 \bullet 12H_2O$, 1.0gm SDS, and 1.39ml H_2SO_4 in one liter of water. Just prior to assay, reagents I and II were mixed 1:1 (v/v) and 0.2ml of the mixed reagent was added to 0.2ml of sample; the sample was then heated for 15 minutes at 100°C in a water bath. The sample was cooled and 0.5ml of reagent III was added and mixed. The OD_{690nm} was then determined.

Total Carbohydrate Determination- Total carbohydrate was determined by mixing known volumes (10-50ul) of samples with 50ul of 80% phenol followed by the rapid addition of 5.0ml of concentrated sulfuric acid. The sample was then mixed vigorously and incubated at room temperature for 30 minutes prior to the measurement of OD_{490nm} (Dubois et al., 1956).

UDP-Glucose Pyrophosphorylase Assay- Overnight cultures of each strain were transferred to 100ml of fresh medium A/0.2% glucose and grown to late log phase at either 30°C or 42°C as indicated. Cells were harvested by centrifugation (10,000xg, 4°C for 10 minutes) and washed two times in cold 0.9% saline. All remaining steps were performed at 4°C. Accordingly, the washed cells were resuspended in 2.0ml of 0.1M Tris-HCl/10mM dithiothreitol (DTT), pH 7.5, and disrupted by sonication with a Sonifier Cell Disrupter using a microtip probe (Heat Systems-Ultrasonic, Inc). Cell membranes and debris were removed by centrifugation at 130,000xg for 90 minutes at 4°C. The supernate solution (S130) was kept on ice and assayed immediately. The enzyme activity was determined by method C of Nakae and Nikaido (Nakae and Nikaido, 1971). Known volumes of S130 (25-100ul) were added to 0.5ml of reaction mixture (50mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, 3mM cysteine, 0.4 umole NADP, 2 nmole glucose-1,6-diphosphate, 0.03 units phosphoglucomutase, 0.14 unit glucose-6-phosphate

dehydrogenase, and 0.5 umole UDP-glucose). The reaction was started by the addition of 15ul of 0.1M sodium pyrophosphate and the OD_{340nm} was monitored at room temperature. The extinction coefficient for all NADP-dependent reactions was 6.22 $cm^{-1}umole^{-1}$.

TDP-Glucose Pyrophosphorylase Assay- Known volumes of S130 were added to 0.5ml of reaction mixture (50mM Tris-HCl, pH 7.8, 5mM $MgCl_2$, 0.4 umole NADP, 2 nmole glucose-1,6-diphosphate, 0.03 units phosphoglucomutase, 0.14 unit glucose-6-phosphate dehydrogenase, and 0.5 umole TDP-glucose). The reaction was started by the addition of 20ul of 0.2M sodium pyrophosphate and the OD_{340nm} was monitored at room temperature (Nakae and Nikaido, 1971).

Phosphoglucose Isomerase (PGI) Assay- S20 supernates were obtained from overnight cultures grown in medium B at 37°C. The cells were harvested at 10,000xg for ten minutes at 4°C and washed with 50mM Tris-HCl, pH 7.6/10mM $MgCl_2$. The washed cells were resuspended in 0.5ml of the same buffer and disrupted at 0-4°C by sonication with a Sonifier Cell Disrupter using a microtip probe. Cell membranes and debris were removed by centrifugation at 20,000xg for 30 minutes at 4°C. The supernate solutions (S20) were kept on ice and assayed immediately. The PGI activity was determined by mixing 50-100ul of S20 in 0.95ml of 50mM Tris-HCl, pH 7.6,10mM $MgCl_2$, 200uM NADP, and 0.4U/ml glucose-6-phosphate dehydrogenase and the OD_{340nm} was monitored. After one minute,10ul of 10mM fructose-6-phosphate (F6P) was added and the OD_{340nm} was monitored for an additional two minutes. The rate of NADPH formation in the

absence of F6P was subtracted from the rate observed in the presence of F6P in order to determine activity (Fraenkel and Horecker, 1964).

TDP-Glucose Oxidoreductase Assay- S20 supernates (100ul) of each strain were mixed with a reaction mixture containing 100ul 0.5M Tris-HCl pH 8.0, 100ul 3mM TDP-glucose and 0.5ml of water. Blanks contained an additional 0.3ml of 0.5M NaOH. All sample reaction mixtures were incubated for 30 minutes at 37°C, and the reactions were terminated by the addition of 0.3ml of 0.5M NaOH. The tubes were then incubated for an additional 10 minutes at 37°C, cooled to room temperature and the OD_{320nm} was determined. An extinction coefficient of 6.5 $cm^{-1}umole^{-1}$ was used for calculating activities after subtracting blank values (Zarkowsky and Glaser, 1969).

Phosphomannose Isomerase Assay- S175 supernates were assayed for phosphmannose isomerase (PMI) by modification of a previously published assay (Nikaido et al., 1966). The assay mixture contained the following: 1.0ml 0.1M Tris-HCl, pH 8.0, 1.37ml water, 200ul 1mM NADP, 200ul 100mM $MgCl_2$, 2units phosphoglucose isomerase and 3units glucose-6-phosphate dehydrogenase. Fifty microliters of S175 was added to 1.0ml of assay mixture, and the OD_{340nm} was monitored to establish baseline activity. Reactions were initiated by the addition of 50ul of 0.1M mannose-6-phosphate, and the initial rate at OD_{340nm} was recorded.

Glucose-6-phosphate Dehydrogenase Assay- The glucose-6-phosphate dehydrogenase assay was carried out in a manner identical to that described for the PGI assay, with the exception that 10ul of 14mM glucose-6-

phosphate was substituted for fructose-6-phosphate in order to start the reaction (Fraenkel and Horecker, 1964).

Radiochemicals and Reagents

All antibiotics as well as GlcNAc, UDP-glucose, UDP-GlcNAc, TDP-glucose, glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate, glucose-1,6-diphosphate, NADH, NADP, dithiothreitol, chitobiose, chitotriose, chitotetraose, α-methyl-mannopyranoside, 4-chloro-1-naphthol, N-octylglucoside, and 3,3'-diaminobenzidine-4HCl were purchased from Sigma (St. Louis, Mo). GDP-Mannose, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, and phosphoglucomutase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Polyclonal anti-O8 antiserum (Lot#4) was purchased from Penn State University (University Park, Pa). Goat-anti-mouse-IgG peroxidase conjugate was purchased from Kirkgaard & Perry Laboratories, Inc. (Gaithersburg, Md.). Purified LPS standards were obtained from List Biological Laboratories, Inc (Cambell, Calif). Radioisotopes were purchased as follows: [2-^{3}H]glycerol (200mCi/mmole), [2-^{3}H]mannose (23Ci/mmole), GDP-[2-^{3}H]mannose (24.3 Ci/mmole), from Dupont, New England Nuclear Research Products (Boston, Mass.); [1-^{3}H]GlcNAc (11.2Ci/mmole), from Amersham Corp. (Arlington Heights, Ill.); and L-[^{35}S]methionine (1051Ci/mmole) from ICN Pharmaceuticals, Inc (Irvine, CA.). Readi-Safe scintillation cocktail was purchased from Beckman Instruments Inc. (Fullerton, Calif.). Fractogel-TSK was purchased from Supleco Inc. (Bellefonte, Pa.).

RESULTS

ECA-trace Strain Construction

The original *S. typhimurium* ECA-trace strains were constructed by conjugation of the *rfe-rff* region into *rff* Δ*rfb* mutants using *S. typhimurium* strain SA464 (HfrK1-2, *rfe-rff*$^{+}$) as donor (Rick et al., 1988; Mäkelä et al., 1976). Hfr strains are created when insertion elements present in the F plasmid allow the plasmid to integrate into the host cell chromosome at random sites in a mechanism similar to transposons. Unfortunately, the F factor of HfrK1-2 inserts into the *rfa* gene cluster resulting in an Rfa or "rough" phenotype (Sanderson et al., 1972). More specifically, the phage-sensitivity pattern of the HfrK1 strain indicates a defect in the *rfaG* locus which is the structural gene for the glucose I transferase (Sanderson and Saeed, 1972). Accordingly, this defective gene would be transferred at a 100% frequency during conjugation, as it would the first host gene transferred due to its close proximity to the inserted F factor origin of transfer. This now interrupted gene if recombined in the F^{-} recipient would produce a strain synthesizing an LPS having an Rd_1-chemotype. Strains possessing an Rd_1-chemotype show increased sensitivity to hydrophobic agents (Roantree et al., 1977; Sanderson et al., 1974). Therefore, in order to examine the basis for the sensitivity of ECA-trace strains to hydrophobic agents, it became necessary to construct new ECA-trace strains using an Hfr with a different F factor insertion point. *S. typhimurium* strain SA975

(HfrK13, *rfe-rff*$^+$) was chosen for this purpose since the origin of transfer is located at 78 minutes and transfer of genetic loci occurs in a clockwise direction. Accordingly HfrK13 transfers the *rfe-rff* region at a high frequency and has no affect on the synthesis of LPS (Sanderson et al., 1972).

Matings between SH5150 (*ilv, rff,* Δ*his-rfb*,SDS^R) and SA975 (*ilv*$^+$, *rfe-rff*$^+$, SDS^R, *thr, leu,* HfrK13) were performed by conjugation method one and transconjugates possessing an *ilv*$^+$ phenotype were selected on medium C plates supplemented with 1% glucose and 0.1 mM histidine (final concentrations). The threonine and leucine auxotrophies of SA975 served as counter selections for the donor strain since neither threonine or leucine were present in the medium. The transconjugates were screened for his- auxotrophy, and LPS-chemotype by sensitivity to phage Felix-O (Ra chemotype sensitivity). Transconjugates that were unable to grow on medium C without histidine, and sensitive to phage Felix-O were further screened for the ECA-trace phenotype by Western blot analysis. Candidates able to synthesize only trace amounts of ECA were further screened for SDS sensitivity and lipid II accumulation after growth on medium A containing [^{3}H]GlcNAc.

Strain HR104 was found to satisfy all of the above criteria. Accordingly, strain HR104 accumulated lipid II and synthesized only trace amounts of ECA as determined by Western blot analysis. The strain was also sensitive to SDS, and it showed leakiness of the periplasmic enzyme, RNase. Phage-typing revealed this strain was resistant to phages P22, P1 and C21 whereas it was fully sensitive to Felix-O. The resistance to P22 and sensitivity to Felix-O is indicative of an Ra-LPS chemotype (Wilkinson et al., 1972; Rapin and Kalckar, 1971). This was the expected result since the

parent strain SH5150 possesses a Ra-chemotype LPS due to the *his-rfb* deletion. The sensitivity of strain HR104 to Felix-O and its resistance to phages P1 and C21 showed that the LPS core structure of this strain was unaltered as a result of the construction.

Later in these studies, the availability of the bacteriophage ES18h-1 made it possible to transduce the *rfe-rff* region into strain SH5150. ES18h-1 does not use LPS as a receptor; rather, the receptor for this phage is believed to be an outer membrane protein (Kuo and Stocker, 1970). Strain SH5150 was transduced with an ES18h-1 lysate of PR122 (*ilv*$^+$, *rff*$^+$) and *ilv*$^+$ transductants were selected on medium C plates supplemented with 1% glucose and 0.1mM histidine (final concentrations). The transductants were screened for a histidine auxotrophy and for ECA synthesis by the colony-blot procedure. Transductants showing positive but low amounts of ECA as determined by colony blot were further screened for SDS sensitivity. One such transductant, strain HR197, was found to be sensitive to SDS. Strain HR197 was unable to grow in the absence of histidine, and it produced a weak ECA response in the colony-blot assay. In addition, strain HR197 was found to be RNase leaky. This strain also accumulated lipid II and was able to synthesize only trace amounts of ECA as determined by Western blot analysis. The possible advantage of this construction over that of HR104 was that the amount of genetic material transferred during the construction was minimized. Phage ES18h-1 transduces about one minute of chromosome while at least 4 or 5 minutes of genome were transferred by conjugation to construct HR104.

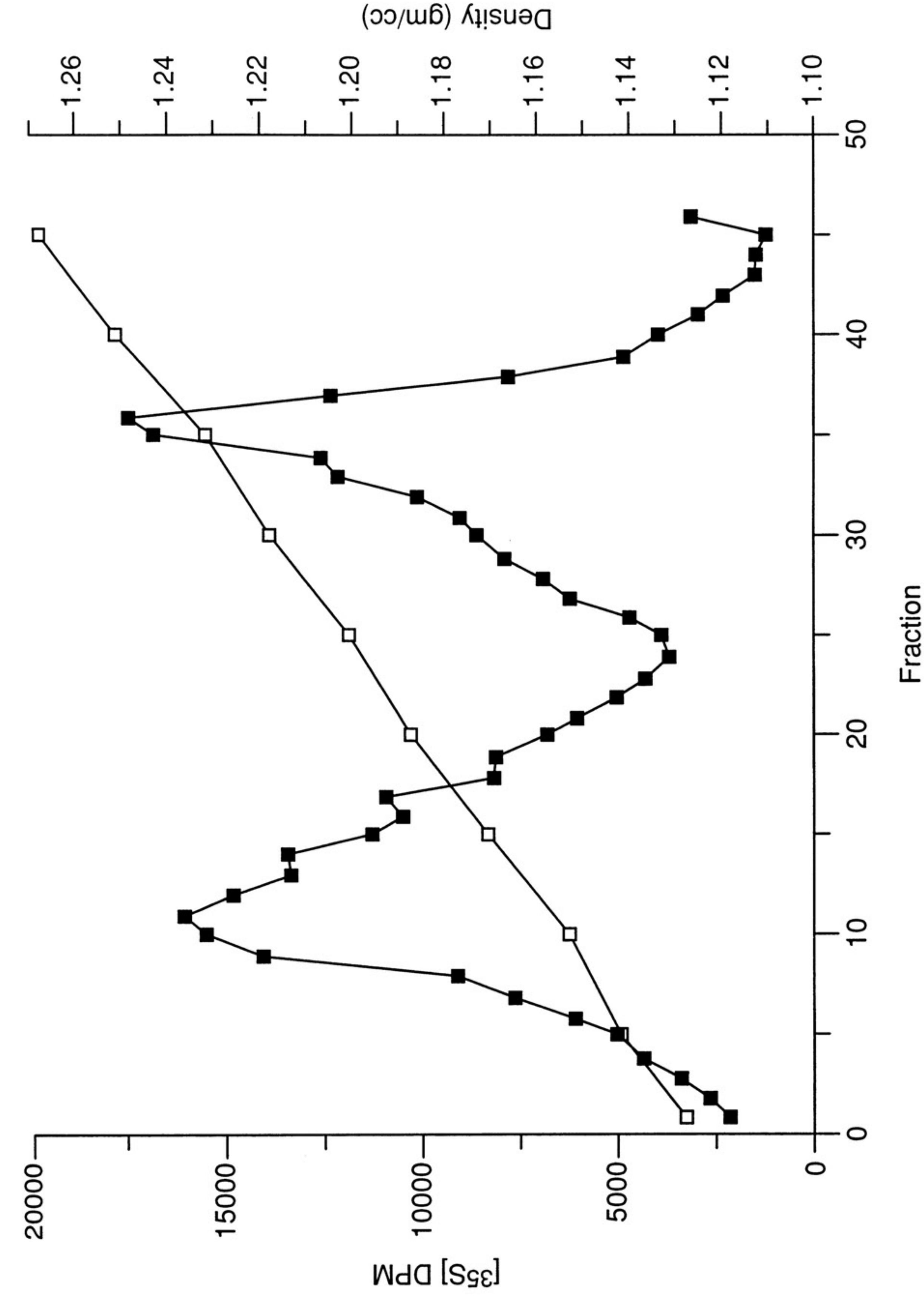
Lipid II Localization

The addition of SDS to cells possessing the ECA-trace phenotype results in lysis (Mäkelä et al., 1976). However, lysis does not occur if these strains are grown in the presence of tunicamycin prior to the addition of SDS (Rick et al., 1988). Tunicamycin inhibits the first step in ECA biosynthesis; i.e., the synthesis of lipid I (Rick et al., 1985). Accordingly, the accumulation of lipid II in ECA-trace strains is also precluded by tunicamycin (Rick et al., 1988). It has also been observed that strain SH5150, from which strains HR104 and HR197 were derived, is a lipid I accumulator and is resistant to SDS. These two observations suggests that the sensitivity of strains HR104 and HR197 to SDS may be related to the accumulation of lipid II. Therefore, the site of lipid II accumulation was determined as an initial step in examining this possibility.

Strains were grown in 200ml of medium C supplemented with 0.2% glucose and amino acids as required. For each group of experiments, tunicamycin was added to a final concentration of 10ug/ml. Growth was maintained at 37°C with aeration. Membranes were uniformly labeled by the addition of 100 uCi [^{35}S]methionine at the beginning of the growth cycle. ECA intermediates were labeled by the addition of 150uCi [^{3}H]GlcNAc to mid-log phase cultures (OD_{600nm} of 0.6) and the cultures were allowed to grow for an additional 30 minutes. Membranes were prepared as described in the Methods section. Figure 5 shows the membrane separation profile for the ECA-trace strain HR104.

Pooled isolated membrane fractions were extracted with chloroform/methanol (3:2, v/v) in order to isolate and identify lipid-linked ECA intermediates. There were significantly more total counts associated

Figure 5. Sucrose Density Gradient Profile of HR104 Membrane Separation. Total membranes were labeled and prepared as described in the Methods. The final gradient was fractionated from the top of the tube. The peak corresponding to L1-L2 inner membrane fractions (Osborn et al., 1972) is located at fraction 11. The peak corresponding to the H band or outer membrane fraction (Osborn et al., 1972) is located at fraction 36. Symbols: ■, radioactive counts due to [^{35}S]methionine incorporation into membrane proteins; □, buoyant density.



Density (gm/cc)
1.26
1.24
1.22
1.20
1.18
1.16
1.14
1.12
1.10
Fraction
0
10
20
30
40
50
[35S] DPM
20000
15000
10000
5000
0

Table 2. Incorporation of $[^3H]$GlcNAc into Membrane Fractions

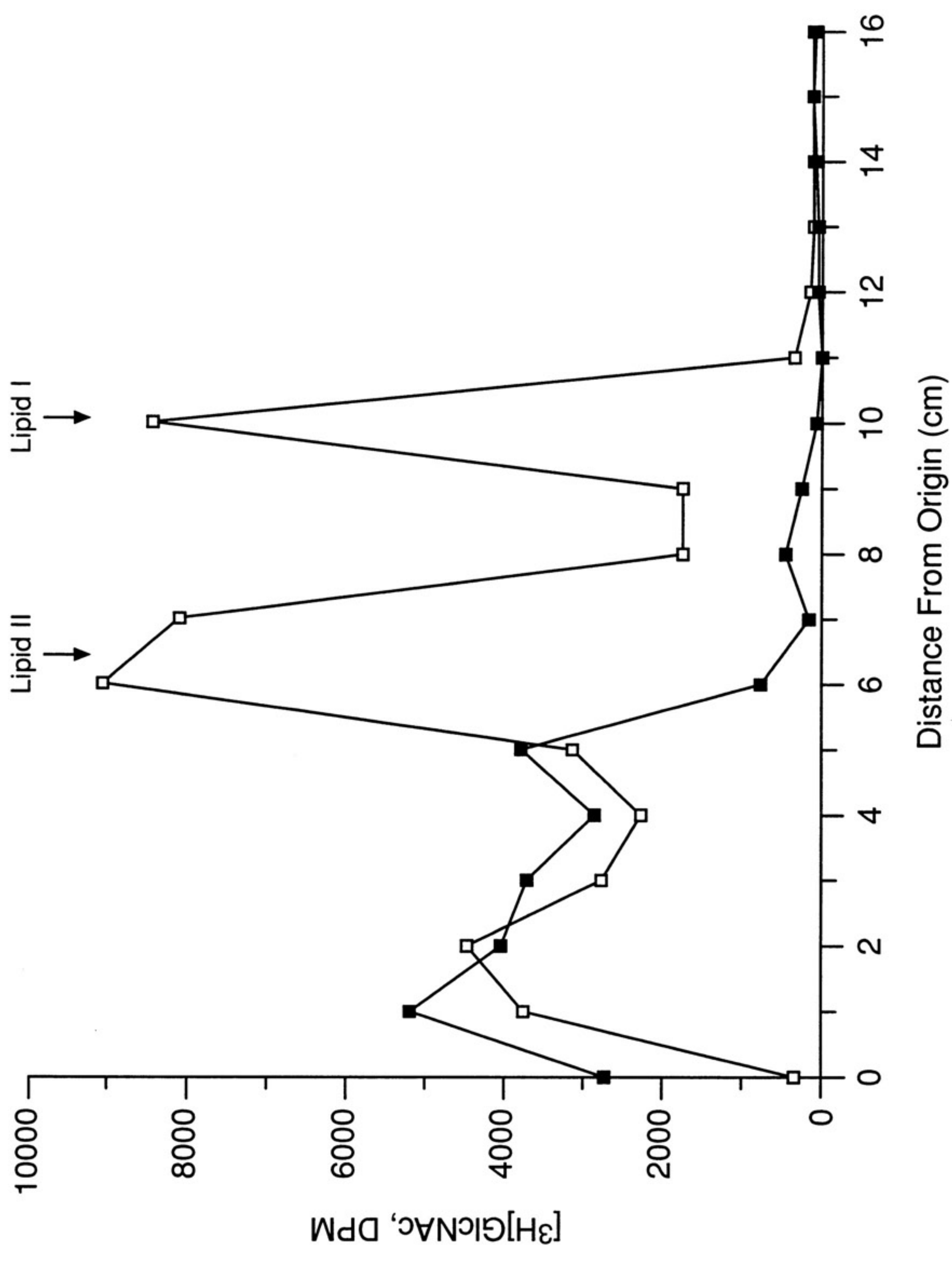
Strain	Membrane Fraction	Total Counts	Total Radioactivity Extracted into $CHCl_3$/MeOH *
SH4892			
(ECA+)	inner	9.9×10^5	6.2×10^4 (6.3)
	outer	4.7×10^6	5.9×10^4 (1.3)
SH5150			
(ECA-)	inner	1.3×10^6	9.0×10^4 (6.9)
	outer	7.2×10^6	9.0×10^4 (1.2)
HR104			
(ECA"trace")	inner	8.0×10^5	1.4×10^5 (17.5)
	outer	4.9×10^6	8.2×10^4 (1.7)

* Values in parentheses indicate the percentage of total counts extracted into $CHCl_3$/MeOH (3:2, v/v).

with the outer membrane fractions due to $[^3H]$GlcNAc incorporation into LPS and peptidoglycan which remains associated with the outer membrane fraction (Table 2). However, the chloroform/methanol extractable counts were approximately equal for both membrane fractions. The identity of radioactive components present in the chloroform/methanol extracts was analyzed by SG81 paper chromatography. Figure 6 shows the chromatographic profile obtained by analysis of HR104 inner and outer membrane extracts by SG81 paper chromatography. Radioactive peaks were tentatively identified based on the similar migration of partially purified lipid I and lipid II standards present on the same chromatograms. The data (Table 3) indicates that lipid II accumulates in the inner membrane of HR104. Data obtained for the control strain SH5150 (ECA-) revealed markedly less radioactivity in the lipid II area of the chromatogram and essentially no radioactive lipid II was found in extracts of strain SH4892 (ECA+). SH5150 is the parent strain of HR104 and the *rff* defect in this strain results in the accumulation of lipid I (Rick et al., 1988). The amount of radioactivity located in the lipid II region of the chromatogram extracted from HR104 inner membranes was reduced to SH5150 levels when HR104 was grown in the presence of tunicamycin. The data show that lipid I also accumulates in the inner membrane of strain SH5150.

The inner and outer membrane fractions were identified by their buoyant densities; 1.14-1.16gm/cc for the inner membrane and 1.22gm/cc for the outer membrane (Osborn et al., 1972). The fractions were also assayed for NADH oxidase activity and KDO which serve as markers for the inner and outer membranes, respectively (Osborn et al., 1972; Osborn and Munson, 1974). The NADH oxidase activity was found to be localized to the

Figure 6. SG81 Chromatography of HR104 Membrane Extracts. Membrane fractions were extracted with $CHCl_3$/MeOH (3:2, v/v) and the extracts were applied to SG81 paper. Chromatograms were developed in solvent B and prepared for counting as described in the Methods section. □, inner membrane extract; ■, outer membrane extract. Lipid I and lipid II standards were partially purified by DEAE-chromatography as detailed in the Methods section, and they were applied to separate lanes on the SG81 chromatogram. Rf values for lipid I and lipid II were determined to be 0.62 and 0.40 respectively.



Lipid II
Lipid I
[3H]GlcNAc, DPM
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
12
14
16
Distance From Origin (cm)

Table 3. Localization of [^{3}H]GlcNAc-labeled Lipid II in Cell-Envelope Membrane Fractions

STRAIN	Membrane Fraction	Radioactivity *(dpm) Recovered in: Lipid II	Lipid I
SH4892 (ECA+)	inner	81 (54)	25 (63)
	outer	42 (38)	33 (37)
SH5150 (ECA-)	inner	5891 (3969)	12168 (11350)
	outer	1675 (1121)	1637 (942)
HR104 (ECA"trace")	inner	17206 (6623)	84 (111)
	outer	942 (2480)	50 (59)

* Values in parentheses indicate radioactivity recovered after cells were pretreated with tunicamycin.

Table 4. Membrane Markers

Membrane Fraction	NADH Oxidase[a]	KDO[b]
Inner	500 (23.3)[c]	11.7
Middle	139	38.7
Outer	17.3	111

[a] Activity expressed as nmol/min/mg of protein

[b] Values expressed as ug KDO/mg of protein

[c] Value in parentheses is NADH Oxidase activity after 16mM KCN was added.

NADH Oxidase and KDO were assayed as described in the Methods. Protein was assayed by the BCA method.

inner membrane fractions while KDO content was ten fold higher in the outer membrane fractions (Table 4).

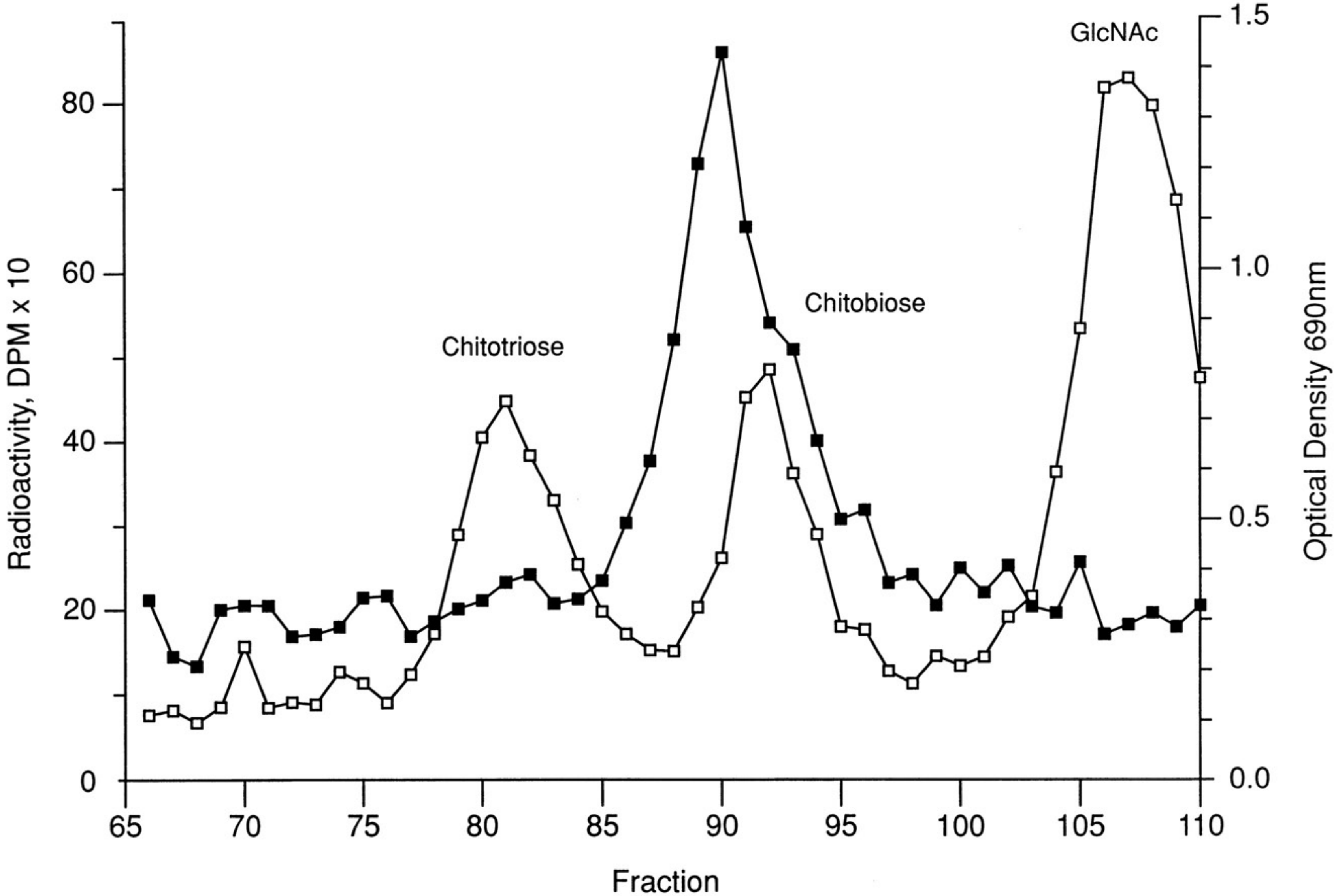
The lipid II isolated from HR104 inner membranes was further characterized by examining the size of the carbohydrate portion by P2-gel filtration chromatography after mild-acid hydrolysis of the putative lipid II peak eluted from the SG81 chromatogram. The inner membrane fraction obtained from HR104 cells labeled with [^{3}H]GlcNAc was extracted, and the extract was subjected to SG81 paper chromatography. The radioactive area corresponding to lipid II was eluted and treated with mild acid, and the water soluble fraction was subjected to P2 gel filtration chromatography as described in the Methods section. The radiolabeled water-soluble material released by mild acid eluted in the same fractions as chitobiose (Figure 7).

ECA Localization

ECA is reported to be localized to the outer membrane (Kuhn et al., 1988). Since ECA-trace strains accumulate lipid II in the inner membrane, experiments were conducted to determine if the translocation and localization of the trace-ECA was altered in these strains.

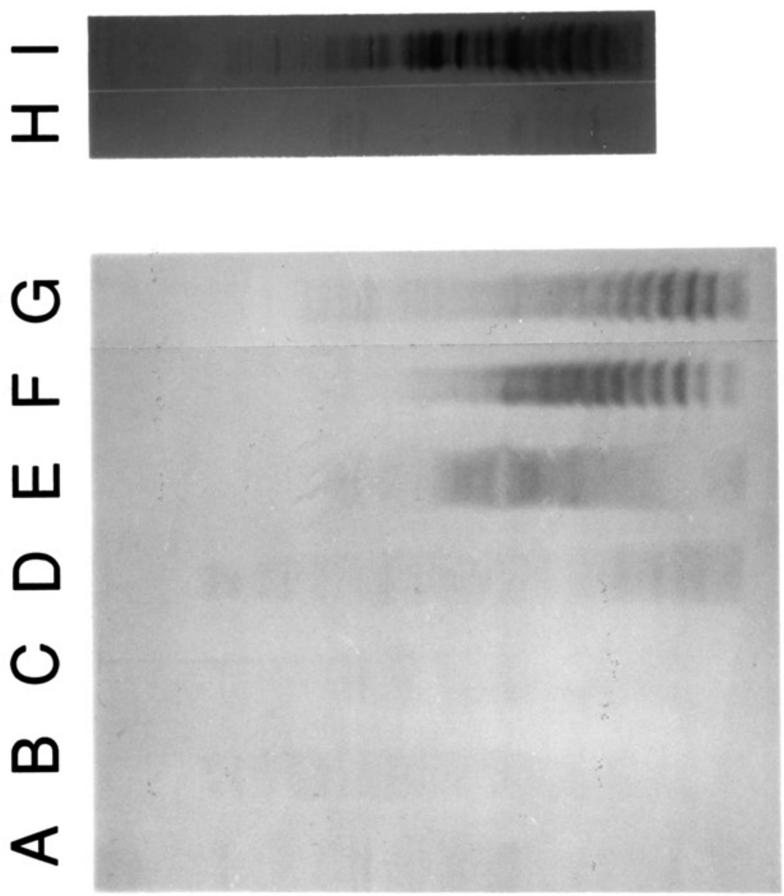
Membranes from strains HR104 and LT2 (wild type) were prepared and the isolated inner and outer membranes were analyzed for ECA by Western blot analysis using anti-ECA monoclonal antibody. The data from these analyses revealed that both ECA and trace-ECA were associated only with the inner membrane fractions of both strains (Figure 8). Two additional strains PR122 and SH5150 were also analyzed. Analysis of strain PR122 also revealed that ECA was only associated with the inner membrane fraction. No

Figure 7. Bio-Gel P2 Chromatography of the Radioactive Component Released from Lipid II by Mild-Acid Hydrolysis. [^{3}H]GlcNAc-labeled lipid II was extracted from the isolated inner membrane fractions and the extracts were applied across the loading area of a SG81 paper, dried and developed in solvent B. A section of the chromatogram was fractionated and counted to locate the lipid II peak. The remaining area of the paper containing the peak was eluted with solvent C and collected. The solvent was removed under a stream of nitrogen and the residue was mild-acid hydrolyzed and subjected to Bio-Gel P2 chromatography as described in Methods. Radioactivity was determined by counting 0.5ml of each fraction. GlcNAc (N-acetyl-glucosamine), chitobiose ($GlcNAc_2$) and chitotriose ($GlcNAc_3$) standards (1 umole each) were added to the the sample prior to loading on the column. The elution pattern of the standards was determined by assaying 200ul of each fraction by the ferricyanide-reducing sugar assay (Methods). Symbols: ■, radioactivity; □, OD_{690nm}



Radioactivity, DPM x 10
0
20
40
60
80
Optical Density 690nm
0.0
0.5
1.0
1.5
65
70
75
80
85
90
95
100
105
110
Fraction
Chitotriose
Chitobiose
GlcNAc

Figures 8. ECA-Membrane Localization as Determined by Western Blot Analysis. Strains LT2 (wild type), PR122 (Rc-LPS, ECA^+), HR104 (ECA-trace) and SH5150 (ECA^-) were grown in 200ml of medium A to an OD_{600nm} of 0.9. Membranes were uniformly labeled during the growth phase by the addition of 100uCi [2-^{3}H]glycerol (200mCi/mmole). The membranes were prepared and isolated as described in the Methods section. The inner and outer membrane fractions were identified by their buoyant densities as well as by the location of NADH oxidase and KDO. The total protein was determined for the final pooled membrane fractions by the BCA assay (Methods). The membrane fractions were pelleted and resuspended in electrophoresis sample buffer to a concentration of 200ug protein per 100ul. Samples were then prepared for Western blot analysis as described in the Methods section. Lane A, SH5150 outer membrane; Lane B, SH5150 inner membrane; Lane C, HR104 outer membrane; Lane D, HR104 inner membrane; Lane E, LT2 outer membrane; Lane F, LT2 inner membrane; Lane G, ECA Standard; Lane H, PR122 outer membrane; Lane I, PR122 inner membrane.


A B C D E F G
H I

ECA was detected in either membrane fraction when strain SH5150 (ECA-) was analyzed in a similar manner.

Antibiotic Sensitivities of ECA-Trace Strains

ECA-trace strains have previously been reported to be sensitive to several hydrophobic agents (Mäkelä et al., 1976). However, this report provided only qualitative information on a limited number of agents. Therefore, it was felt that a quantitative comparison of the effects of several of these agents on the growth of mutants of *S. typhimurium* defective in ECA and LPS synthesis would perhaps provide insight as to the nature of the ECA-trace strain membrane defects. The MIC results are divided into three groups based on the sensitivity of the ECA-trace strain HR104 to the various agents. These results are summarized in Tables 5-7. Partition coefficients for each agent were calculated based on the following relationship:

$$\frac{\text{Solubility of Agent in Chloroform}}{\text{Solubility of Agent in Water}} = \text{Partition Coefficient}$$

Solubility values were taken from the literature (Weiss et al., 1957). Unlisted solubilities were determined by dissolving each agent to saturation in chloroform and in water, spinning out the undissolved material, and then transferring 1.0 ml of the saturated solution to a tared tube. The solvent was then evaporated at 100°C, and the residue was weighed (Weiss et al., 1957). With some exceptions, agents with low partition coefficients had less of an effect on the growth and viability of the ECA-trace strain HR104 than agents with high partition coefficients. Detergents also had lower MICs for the ECA-

Table 5: MICs: Agents that all Strains Show Relatively Equal Response.

			Minimal Inhibitory Concentration (ug/ml)						
		Strain:	SH4892	HR104	SL3769	G30A	LT2	G30	SH5150
AGENT	M.W.	P.C. Chemotype*	Ra/+	Ra/tr	Rd1/+	Re/+	wt	Rc/+	Ra/-
Amp	371.4	<0.01	4.7	2.4	9.4	4.7	4.7	4.7	4.7
PenG	372.5	0.02	5.9	2.9	5.9	2.9	5.9	5.9	5.9
Tet	480.9	0.07	0.47	0.47	1.9	0.47	0.94	0.94	0.94
Otet	496.9	0.06	2.8	1.4	0.7	1.4	2.8	2.8	2.8
Carb	378.4	<0.01	5.9	2.9	5.9	5.9	5.9	12	2.9
Ceph	415.4	<0.01	7.5	3.8	7.5	7.5	7.5	15	15
ActD	1255	>20	24	12	94	24	24	24	47

Abbreviations: M.W., molecular weight; P.C., partition coefficient; Amp, Ampicillin; PenG, Pennicillin G; Tet, Tetracycline; Otet, Oxytetracycline; Carb, Carbenicillin; Ceph, Cephalosporin; ActD, Actinomycin D

* designations in parentheses indicate either the presence of trace levels of ECA (Tr) or the presence(+) ar absence (-) of wild-type levels of ECA

Table 6: MICs: Increased Sensitivity of The ECA-trace Strain Relative to Control Strains

				Minimal Inhibitory Concentration (ug/ml)						
			Strain:	SH4892	HR104	SL3769	G30A	LT2	G30	SH5150
AGENT	M.W.	P.C.	Chemotype*	Ra/+	Ra/tr	Rd1/+	Re/+	wt	Rc/+	Ra/-
Kan	582.6	<0.01		2.8	0.7	1.4	1.4	2.8	2.8	5.6
Ctet	515.4	<0.01		1.9	0.5	0.24	0.5	1.9	1.9	0.94
Van	1486	<0.01		450	113	225	113	450	450	0.9
MalG	365	4.2		60	0.23	0.23	0.12	<60	30	60
Ery	734	>10		75	2.3	4.7	1.2	75	75	75
Bac	1411	0.12		281	70	141	141	1130	563	281
Strep	1457	<0.01		33	8	8	33	33	66	16
C.V.	408	14.4		38	2.3	2.3	0.6	<38	19	38
Clox	458	<0.01		263	66	66	16	263	132	263

* designations in parentheses indicate either the presence of trace levels of ECA (Tr) or the presence(+) or absence (-) of wild-type levels of ECA

Table 6: MICs: Continued

				Minimal Inhibitory Concentration (ug/ml)						
			Strain:	SH4892	HR104	SL3769	G30A	LT2	G30	SH5150
AGENT	M.W.	P.C.	Chemotype*	Ra/+	Ra/tr	Rd1/+	Re/+	wt	Rc/+	Ra/-
Dclox	492	<0.01		188	23	94	5.9	188	188	375
PolyB	1200	0.05		0.38	0.02	0.09	0.75	1.5	0.38	0.18
Novo	635	>20		75	4.7	1.2	0.15	75	38	38
Rif	720	18.8		75	19	19	0.6	75	75	38

Abbreviations: M.W., molecular weight; P.C., partition coefficient; Kan, Kanamycin; Ctet, Chlorotetracycline; Van, Vancomycin; MalG, Malachite Green; Ery, Erythromycin; Bac, Bacitracin; Strep, Streptomycin; C.V., Crystal Violet; Clox, Cloxacillin; Dclox, Dicloxacillin; PolyB, Polymyxin B; Novo, Novobiocin; Rif, Rifamycin

Table 7: MICs: Detergents

				Minimal Inhibitory Concentration (ug/ml)						
			Strain:	SH4892	HR104	SL3769	G30A	LT2	G30	SH5150
AGENT	M.W.	P.C.	Chemotype*	Ra/+	Ra/tr	Rd1/+	Re/+	wt	Rc/+	Ra/-
SDS	288	<0.01		>750	94	23	23	750	750	750
Chol	409	<0.01		45mg	703	703	352	45mg	6mg	11mg
Dchol	414	<0.01		900	113	>900	113	900	900	900
TX	647	1.17		37.5	0.15	9.4	0.15	38	38	38
MBE	462	2.54		23	2.9	2.9	1.5	23	12	23

Abbreviations: M.W., molecular weight; P.C., partition coefficient; SDS, Sodium Dodecyl sulfate; Chol, Sodium Cholate; Dchol, Sodium Deoxycholate; TX, Triton X100; MBE, methylbenzylethonium chloride

* designations in parentheses indicate either the presence of trace levels of ECA (Tr) or the presence(+) ar absence (-) of wild-type levels of ECA

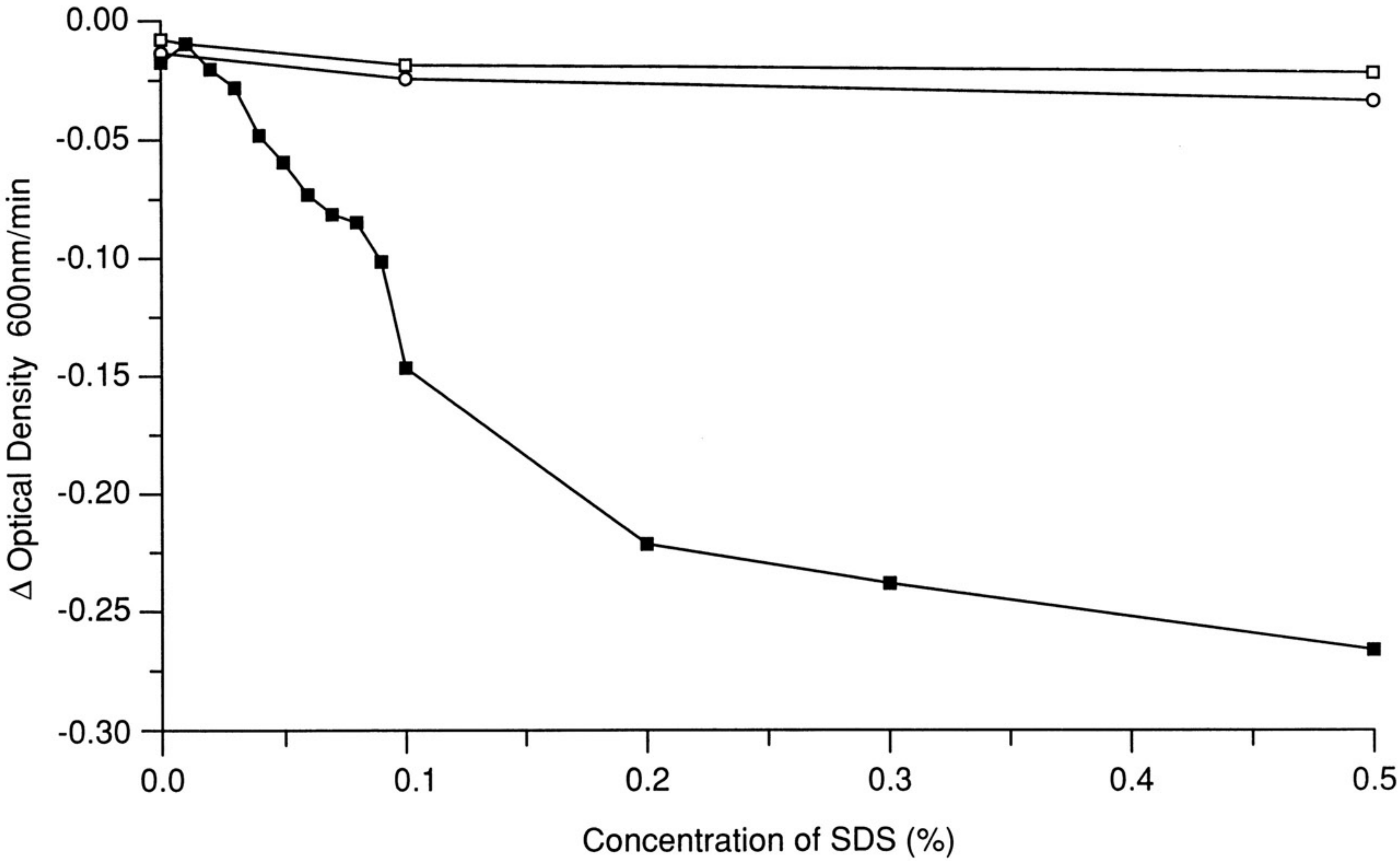
trace strain than for control strains. This pattern was also true for the "deep-rough" LPS strains SL3769 (Rd1) and G30A (Re). In contrast, the wild type (strain LT2), and strains with mutations affecting either the outer core (G30, Rc; SH4892, Ra) or ECA (SH5150, Ra, ECA-), showed similar high-resistance patterns for all hydrophobic agents.

Bacterial Cell Lysis Rate Determinations

It was previously shown that ECA-trace strains are lysed by SDS (0.05%) at 37°C and that this could be reversed by growth of the cells in the presence of tunicamycin (Rick et al.,1988). Additional experiments suggested that the outer membrane of the ECA-trace strain, HR104, is very permeable to SDS as indicated by the rate of cell-lysis as a function of SDS concentration (Figure 9). Furthermore, the addition of 1.0mM EDTA did not significantly increase the sensitivity of strain HR104 to SDS whereas the SDS lysis rates of control strains, strains SH5150 and LT2, were increased in the presence of EDTA (Data not shown).

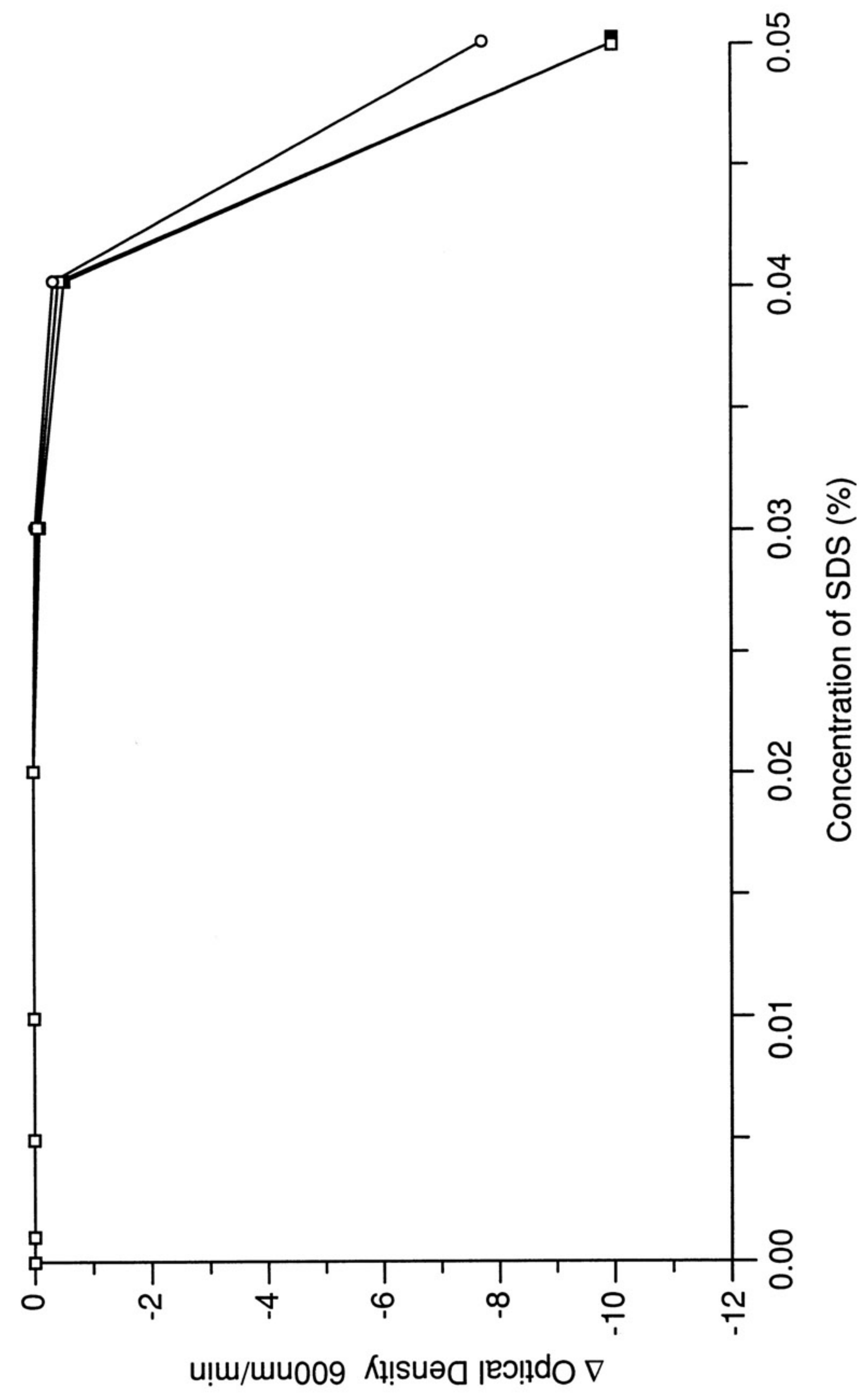
It is possible that the outer membrane is permeable to small amounts of detergents such as SDS that are not normally deleterious to the structural and functional integrity of the cytoplasmic membrane. However, in the case of ECA-trace strains, the accumulation of lipid II may render the cytoplasmic membrane more sensitive to SDS. Accordingly, the effect of SDS on spheroplasts of ECA-trace strains as well as ECA- and wild-type cells was examined to determine if lipid II accumulation had any effect on lysis rates. The rates of SDS-mediated lysis of spheroplasts (Figure 10) did not reveal an increased sensitivity of the cytoplasmic membrane of ECA-trace strains to this detergent. Indeed, the concentration of SDS which resulted in an

Figure 9. SDS-Mediated Lysis of Whole Bacterial Cells. Cells were grown at 37°C to mid-log phase in medium A. SDS was then added at the indicated concentrations (w/v) and the rate of decrease in absorbance at 600_{nm} was determined. Each point represents the average of three initial rate determinations for the indicated SDS concentration. Symbols: ■, HR104 (ECA-trace); □, LT2 (wild type); ○, SH5150 (ECA^-).



0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
Δ Optical Density 600nm/min
0.0
0.1
0.2
0.3
0.4
0.5
Concentration of SDS (%)

Figure 10. Sensitivities of Spheroplasts to SDS-Mediated Lysis. The rate of decrease in absorbance at OD_{600nm} after the addition of various concentrations of SDS was determined for spheroplasts prepared from strains HR104, LT2, and SH5150. Each point represents the average of three rate determinations for the indicated SDS concentration. It should be noted that the error of determining actual lysis rates becomes larger at the higher SDS concentrations due to almost spontaneous lysis, making it almost impossible to get a measurment and any observed differences are not significant. Spheroplasts were prepared according to Osborn et al. (Osborn et al., 1972) as detailed in the Methods, and they were used immediately for lysis experiments. SDS percent concentrations are weight to volume. Symbols: ■, HR104 (ECA-trace); □, LT2 (wild type); ○, SH5150 (ECA^-).



Δ Optical Density 600nm/min
Concentration of SDS (%)
0
-2
-4
-6
-8
-10
-12
0.00
0.01
0.02
0.03
0.04
0.05

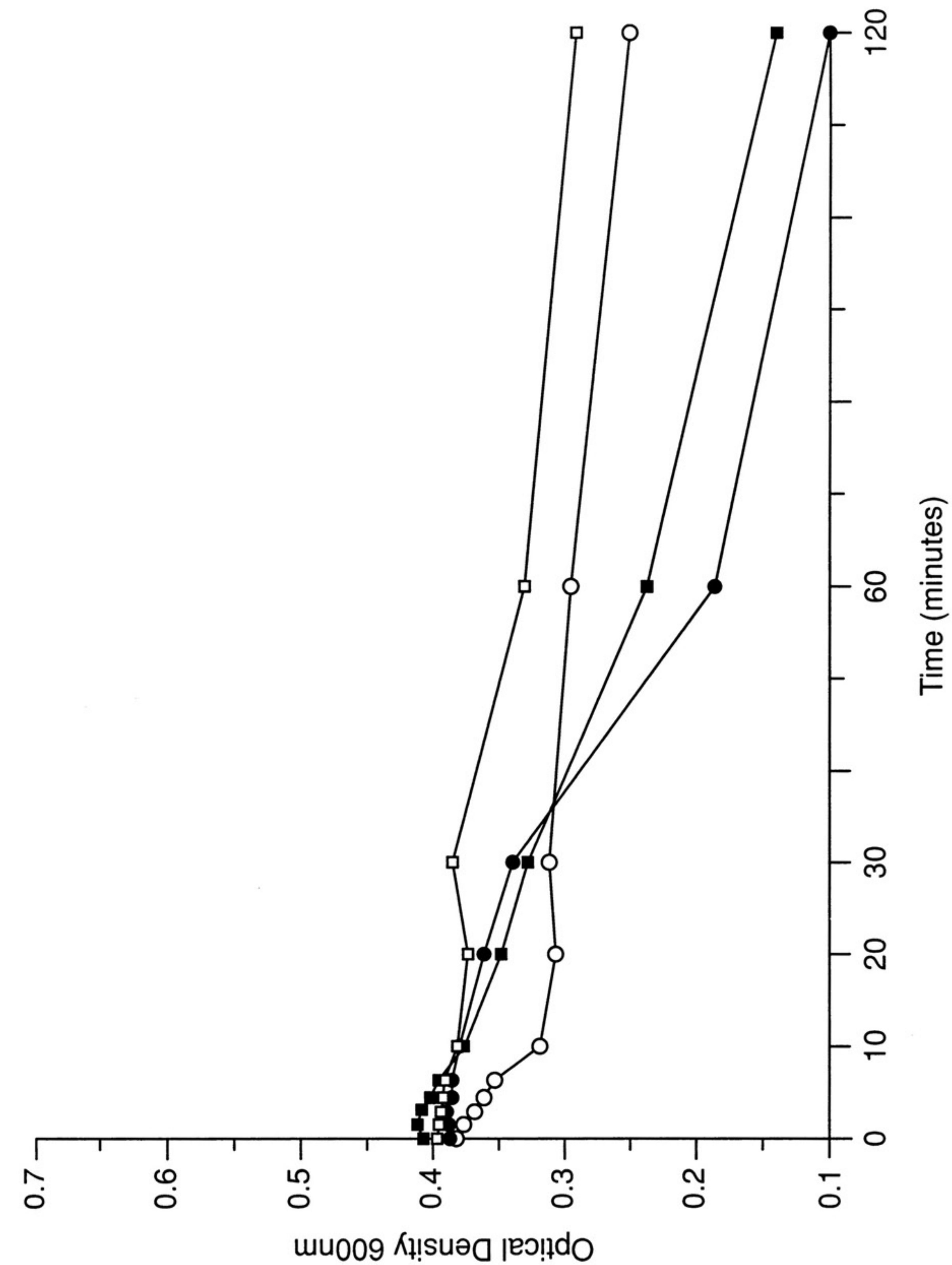
increased rate of lysis of whole cells of ECA-trace strains was very close to the same SDS concentration (0.04-0.05%) which lysed the spheroplasts from all strains tested. These data suggest that the barrier function of the outer membrane of ECA-trace strains has been altered.

E. coli envA mutants have been reported to leak periplasmic contents and they are also sensitive to hydrophobic agents (Young and Silver, 1991). In addition, it was reported that such mutants were susceptible to the action of lysozyme in the absence of EDTA. In contrast, it was reported that "deep-rough" mutants required EDTA to become fully susceptible to the action of lysozyme (Normark et al., 1971). Therefore, to further characterize the outer membrane defect of the ECA-trace strain, the rates of lysozyme-mediated lysis of an ECA-trace strain and control cells was determined in the presence and absence of EDTA. The ECA-trace strain HR104 and the ECA^- parental strain SH5150 were both sensitive to lysozyme, and the presence of EDTA did not significantly enhance lysis (Figures 11 and 12). Analysis of the "deep-rough" strain, G30A (Re), in a similar manner yielded the same results whereas wild type LT2 was only lysed when EDTA was present (Figures 13 and 14).

<u>Construction of an ECA-Minus Mutant of *S. typhimurium* That Accumulates Lipid II.</u>

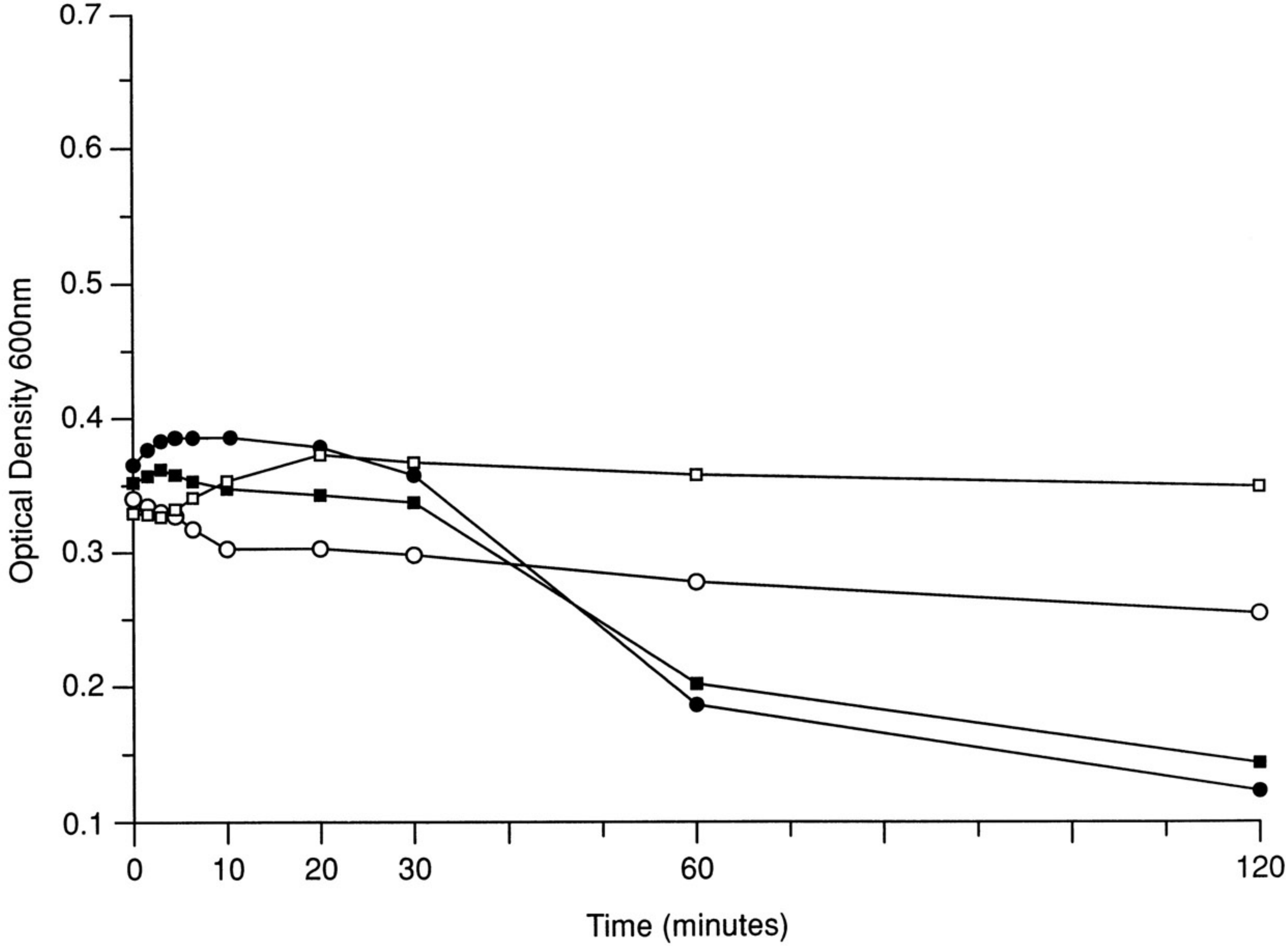
S. typhimurium ECA-trace strains lack the enzymes TDP-glucose pyrophosphorylase and TDP-glucose oxidoreductase due to the deletion of the *his-rfb* region (Lew et al., 1986). These two enzymes are required for the synthesis of TDP-Fuc4NAc, the donor of Fuc4NAc residues in ECA synthesis (Figure 3). One possible explanation for the synthesis of "trace"

Figures 11. Effect of Lysozyme/EDTA on the Lysis of Intact Cells of Strain HR104 (ECA-trace). The absorbance at OD_{600nm} was determined for cells grown to mid-log phase in medium A at various time points after the addition of either100ug/ml (final concentration) of lysozyme, 1mM EDTA, or lysozyme and EDTA. Each point is the average of three determinations. Symbols: □, untreated cells; ■, 100ug/ml lysozyme; ○, 1mM EDTA; ●, 100ug/ml lysozyme and 1mM EDTA.



Optical Density 600nm
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Time (minutes)
0
10
20
30
60
120

Figure 12. Effect of Lysozyme/EDTA on the Lysis of Intact Cells of Strain SH5150 (ECA-). The absorbance at OD_{600nm} was determined for cells grown to mid-log phase in medium A at various time points after the addition of either 100ug/ml (final concentration) of lysozyme, 1mM EDTA, or lysozyme and EDTA. Each point is the average of three determinations. Symbols: □, untreated cells; ■, 100ug/ml lysozyme; ○, 1mM EDTA; ●, 100ug/ml lysozyme and 1mM EDTA.


Optical Density 600nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
60
120
Time (minutes)

931326.12

Figure 13. Effect of Lysozyme/EDTA on the Lysis of Intact Cells of Strain G30A (Re "deep rough"). The absorbance at OD_{600nm} was determined for cells grown to mid-log phase in medium A at various time points after the addition of either 100ug/ml (final concentration) of lysozyme, 1mM EDTA, or lysozyme and EDTA. Each point is the average of three determinations. Symbols: □, untreated cells; ■, 100ug/ml lysozyme; ○, 1mM EDTA; ●, 100ug/ml lysozyme and 1mM EDTA.

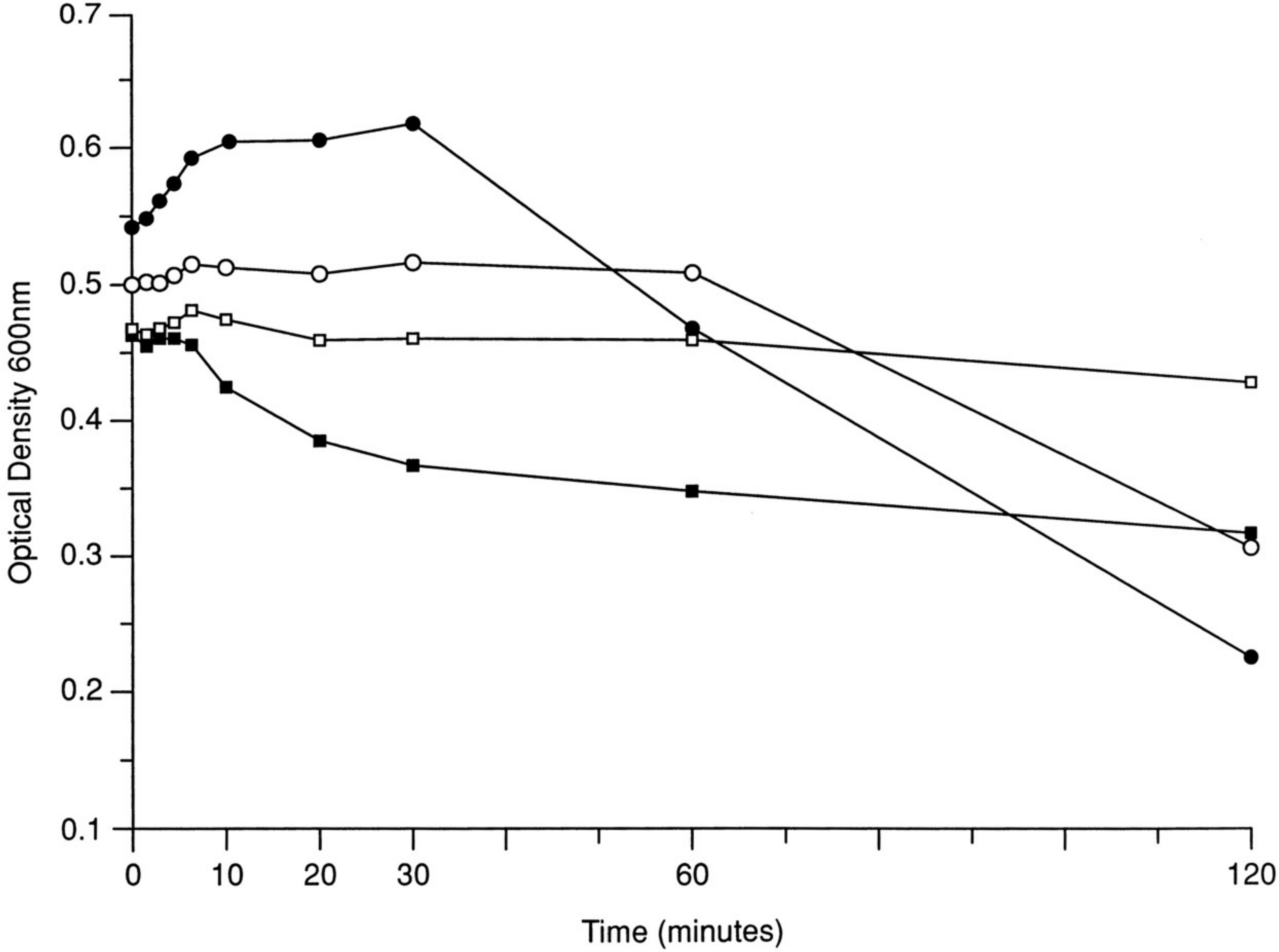
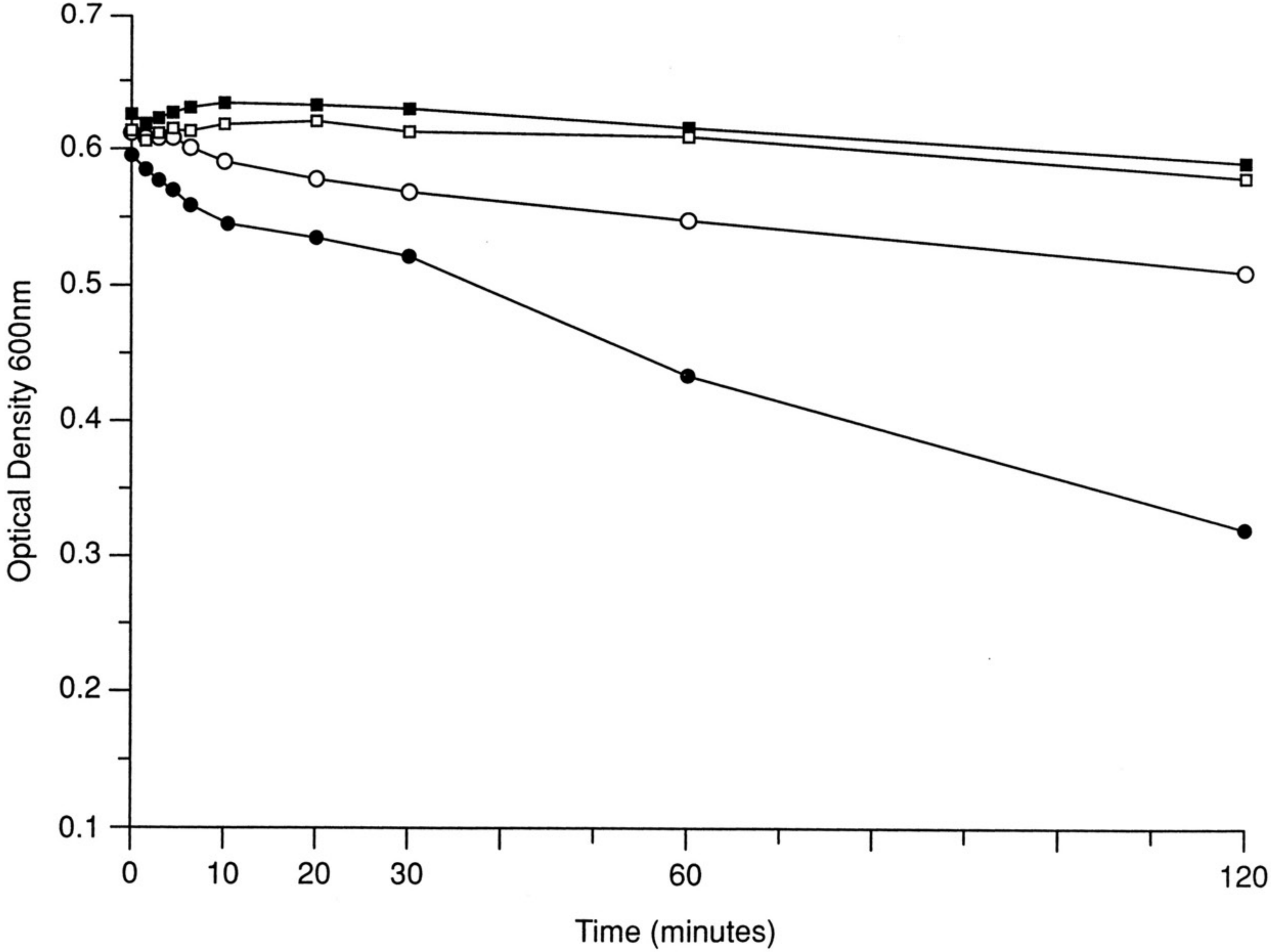

0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical Density 600nm
0
10
20
30
60
120
Time (minutes)

Figure 14. Effect of Lysozyme/EDTA on the Lysis of Intact Cells of Strain LT2 (wild type). The absorbance at OD_{600nm} was determined for cells grown to mid-log phase in medium A at various time points after the addition of either 100ug/ml (final concentration) of lysozyme, 1mM EDTA, or lysozyme and EDTA. Each point is the average of three determinations. Symbols: □, untreated cells; ■, 100ug/ml lysozyme; ○, 1mM EDTA; ●, 100ug/ml lysozyme and 1mM EDTA.


Optical Density 600nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
60
120
Time (minutes)

amounts of ECA is that a small amount of TDP-glucose is produced in vivo by the enzyme UDP-glucose pyrophosphorylase (Lew et al., 1986). Indeed, this proposal was based on the observation that purified UDP-glucose pyrophosphorylase from *S. typhimurium* is able to catalyze the synthesis of TDP-glucose in vitro (Nakae and Nikaido, 1971). In this event, the transfer of a defective UDP-glucose pyrophosphorylase gene (*galU*) into the ECA-trace strain background might completely preclude the synthesis of ECA. In order to test this possibility, a *galU*^{ts} derivative of strain HR104 (HR114) was constructed as shown in Figure 15. The effects of temperature on the ECA phenotype of this derivative were examined along with the activities of UDP-glucose pyrophosphorylase and TDP-glucose pyrophosphorylase at permissive and nonpermissive temperature. Table 8 shows that UDP- glucose pyrophosphorylase and residual TDP-glucose pyrophosphorylase activities for HR114 were nearly abolished at the non-permissive temperature of 42^{o}C. In addition, the strain was found to accumulate lipid II, and it was still able to produce trace amounts of ECA at the non-permissive temperature as determined by SG81 chromatography and Western blot analysis (Figures 16&17). Furthermore, strain HR114 remained SDS sensitive at both permissive and non-permissive temperatures.

Construction of a Phosphoglucose Isomerase-Negative Derivative of Strain HR104.

The inability of the *galU* mutation to abolish the synthesis of trace amounts of ECA raised the possibility that the polymer that was reacting with the anti-ECA monoclonal antibody in ECA-trace strains was structurally different from that of wild type ECA. The immunogenic determinant in ECA

Figure 15. ECA-trace, *galU* Strain Construction. A pool of random Tn10 insertions into the chromosome of *S. typhimurium* strain TT10427 was generated as described in the Methods. P22 transductions into SL1314 were performed at 30°C to allow expression of UDP-glucose pyrophosphorylase and synthesis of a smooth LPS (both UDP-glucose and UDP-galactose are required to complete the LPS-core, and in addition, UDP-galactose is required for O-side chain synthesis- see Figure 2). Phage ES18h-1 was used for all transductions involving rough LPS strains. Transductants at each stage were selected on medium B plates containing 25ug/ml tetracycline. Constructs were screened for their ability to utilize galactose as the sole carbon source at 30°C (permissive) and 42°C (non-permissive). Constructs were also phage typed with P22 and C21 at 30°C and 42°C. The final construct HR114 was gal$^+$ and P22 sensitive at 30°C and gal$^-$ and C21 sensitive at 42°C.

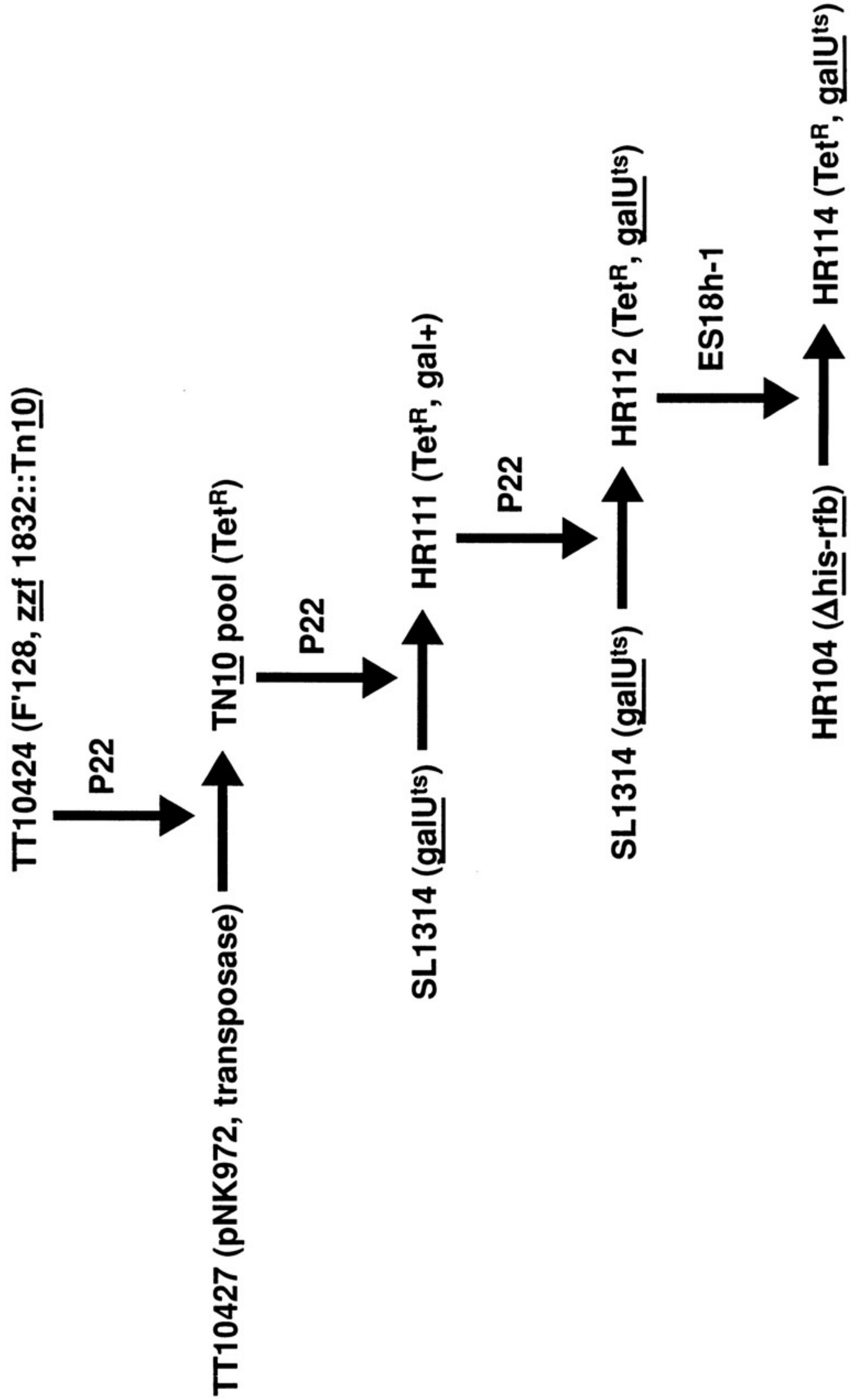

TT10424 (F'128, zzf 1832::Tn10)
P22
TT10427 (pNK972, transposase)
TN10 pool (Tet^R)
P22
SL1314 (galU^{ts})
HR111 (Tet^R, gal+)
P22
SL1314 (galU^{ts})
HR112 (Tet^R, galU^{ts})
ES18h-1
HR104 (Δhis-rfb)
HR114 (Tet^R, galU^{ts})

Table 8: Specific Activities of UDP- and TDP- Glucose Pyrophosphorylase.

UDP-Glucose Pyrophosphorylase Activities

Strain	Relevant Property	Specific Activity * 30°	Specific Activity * 42°
PR122	ECA+	44.4	30.7 (69%)
HR104	"trace"	90.6	57.6 (64%)
HR114	*galU*ts	88.0	6.6 (15%)

TDP-Glucose Pyrophosphorylase Activities

Strain	Relevant Property	Specific Activity * 30°	Specific Activity * 42°
PR122	ECA+	70.1	80.4
HR104	"trace"	2.6	0.4
HR114	*galU*ts	9.5	0

* Specific activity is expressed as nmole/min/mg protein.
Enzymes were assayed as described in the Methods. Protein was assayed by the BCA method. Values in parentheses represent the specific activities at 42°C as a percentage of the activities observed at 30°C for UDP-Glucose Pyrophosphorylase.

Figure 16. Effect of Temperature on the Synthesis of Lipid II by Strain HR114. Strain HR114 was grown in medium A supplemented with 0.2% glucose at 30°C and 42°C. Mid-log phase cells were labeled with [^{3}H]GlcNAc for 30 minutes at the same temperatures and then harvested by centrifugation. The cells were next carried through the extraction procedure for the isolation of Lipid II, and the extracts were analyzed by SG81 paper-chromatography using solvent system B as described in the Methods section. Symbols: □, 30°C (permissive); ■, 42°C (nonpermissive). Lipid II standard was partially purified by DEAE-chromatography as detailed in the Methods section, and it was applied to a separate lane on the SG81 chromatogram.

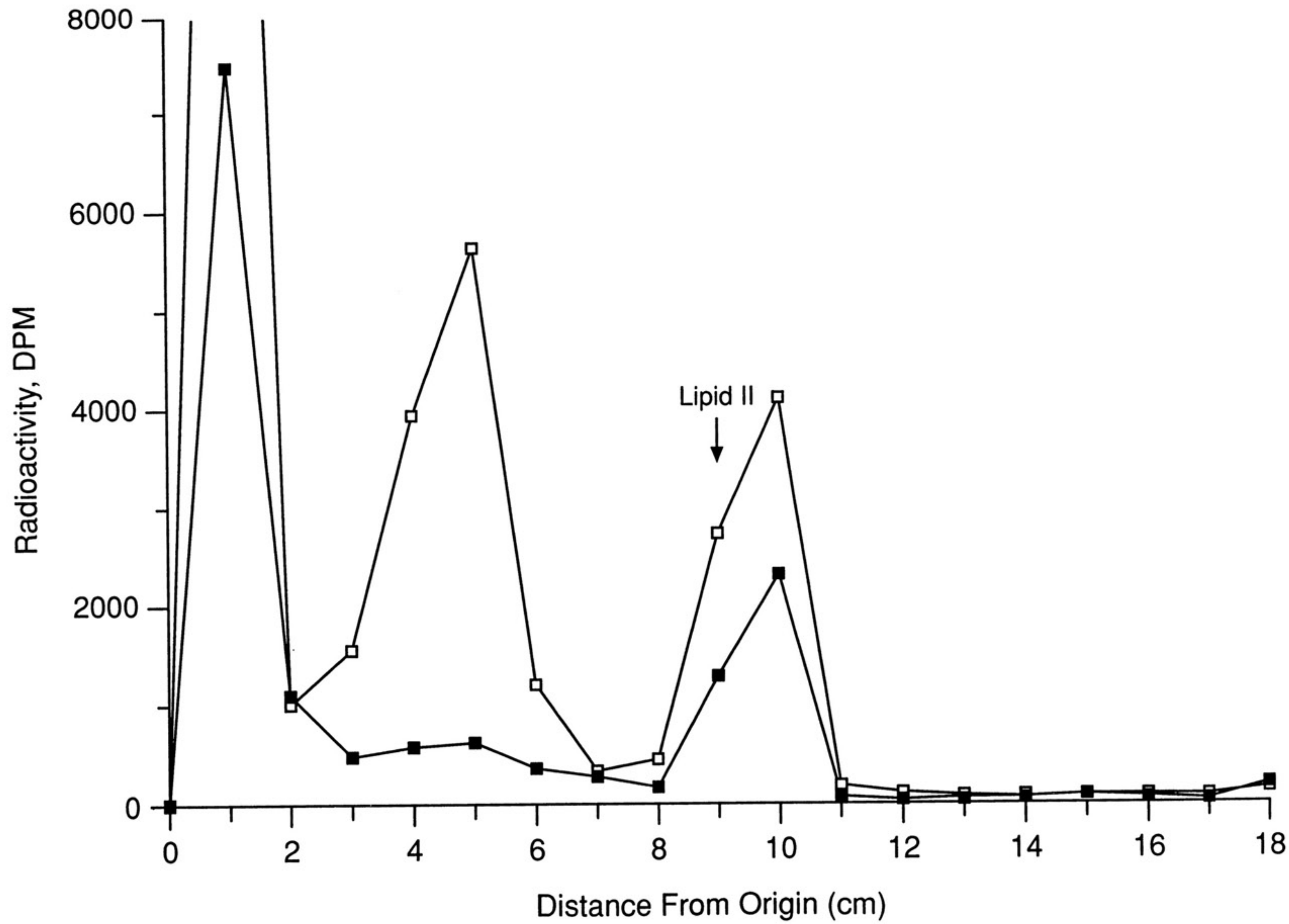
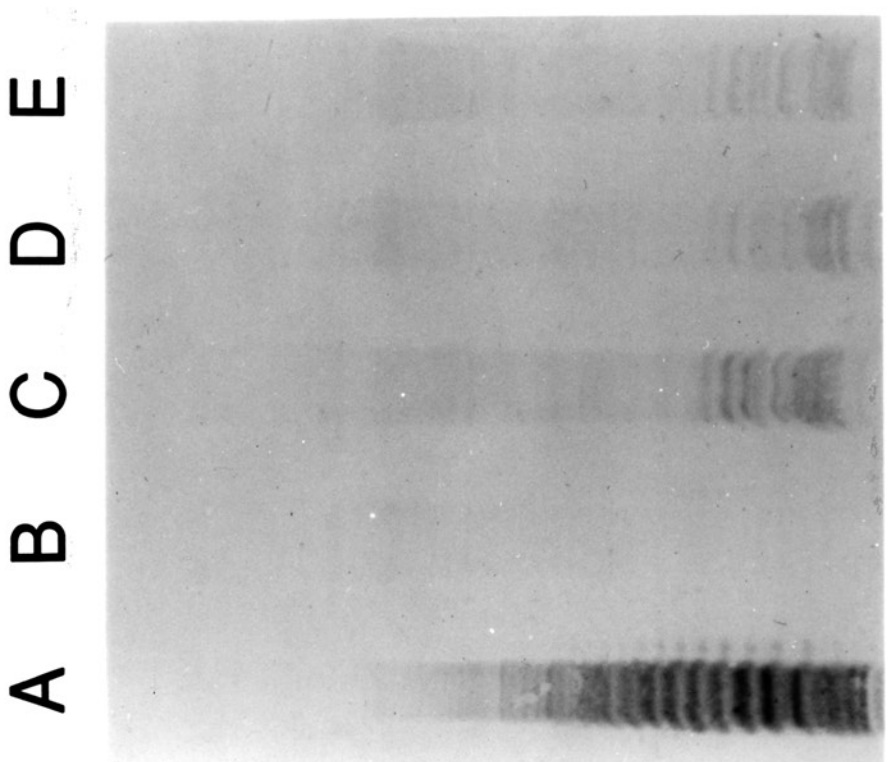

Radioactivity, DPM
8000
6000
4000
2000
0
Lipid II
0
2
4
6
8
10
12
14
16
18
Distance From Origin (cm)

Figure 17. Effect of the *galU* Mutation on ECA as Determined by Western Blot Analysis. Strain HR114 was grown overnight in medium A supplemented with 0.2% glucose at 30°C and 42°C. Strains HR104, SH5150, and PR122 were also grown overnight at 42°C in medium A supplemented with 0.2% glucose. The cells were then harvested and analyzed for the presence of ECA by Western blot analysis as described in the Methods section. Lane A, PR122 (ECA+); Lane B, SH5150 (ECA-); Lane C, HR104 (ECA-trace); Lane D, HR114 (30°C); Lane E, HR114 (42°C).


A
B
C
D
E

that is recognized by the anti-ECA monoclonal antibody used in these studies has been shown to be the ManNAcA residue (Peters et al., 1985). Therefore, the possibility that trace ECA represents a structurally altered ECA that is still able to react with the antibody could not be ruled out. The loss of trace ECA following the insertion of a *pgi* mutation into the ECA-trace background would provide evidence for the involvement of a "glucose" derived sugar component in trace ECA. This follows from the fact that in the absence of exogenous glucose the reaction catalyzed by phosphoglucose isomerase is essential for the synthesis of all glucose-derived metabolites (Figure 3). The initial construction of a derivative of HR104 possessing a "null" mutation in *pgi* necessitated an interspecies transfer of a *pgi*::Tn10 locus from *E. coli* into *S. typhimurium*. This was facilitated by the use of a *S. typhimurium* mismatch-repair mutant, SA3858 (Rayssiguier et al., 1989). The steps involved in the construction of the *pgi*::Tn10-insertion of HR104 are summarized in Figure 18. The final construct, HR159 gave a glucose-minus phenotype on TZ and BTB plates containing glucose. This strain also failed to grow on medium C with glucose as a sole carbon source. The phosphoglucose isomerase activity for strain HR159 was 1 nm/min/mg protein compared to 441 nm/min/mg protein for strain HR104. Glucose-6-phosphate dehydrogenase levels for both strains were normal (30-60 nm/min/mg protein). In addition, both strain HR104 and strain HR159 accumulated lipid II when grown in the absence of glucose (Figure 19). Western blot analyses revealed the absence of trace amounts of ECA in extracts of strain HR159 grown in the absence of exogenously supplied glucose (Figure 20). These results support the conclusion that "trace-ECA" does indeed contain a glucose-derived component. Strain HR159 was also found to be sensitive to SDS when grown in the absence of glucose. In contrast, HR104 was not

sensitive to SDS when grown on medium C, regardless of the carbon source. In addition, the SDS sensitivity of the *pgi* construct cannot be clearly attributed to either the accumulation of lipid II or the presence of "trace-ECA" polymer since the LPS synthesized by strain HR159 in the absence of glucose has an Rd_1 ("deep rough") chemotype. As described previously, "deep rough" strains are inherently sensitive to detergents and other agents. The MICs of a variety of antibiotics and other agents for the trace strain, HR104, and it's *pgi* derivative, strain HR159, were determined in both rich media (medium A) and minimal media (medium C without glucose) in an attempt to identify an agent whose effectiveness was not greatly affected by LPS chemotype or by the composition of the media but was affected by the presence of "trace-ECA" (strain HR104) or the accumulation of lipid II (strain HR159). To aid in the identification of such an agent, strains SL3769 (Rd_1 chemotype), G30A (Re chemotype), SH5150 (ECA-), and LT2 (wild type) were included as controls. These comparisons are summarized in Figures 21-26. Although these results failed to identify a differentiating agent, they did demonstrate that the composition of the media on which the cells were grown had a pronounced effect on the sensitivity of strain HR104 to various agents. More specifically, the data show that HR104 is refractory to most of the tested agents when grown in minimal medium (Figure 21).

Construction of an *rffT* Derivative of Strain HR104

Investigations of the *galU* and *pgi* derivatives of strain HR104 failed to provide a means for ascribing the SDS-sensitivity of strain HR104 to either the accumulation of lipid II or the synthesis of only trace amounts of ECA. In part this was due to the fact that both mutations lead to altered LPS.

Figure 18. Construction of a *pgi* Derivative of Strain HR104. Strains SV107, P72 and HR138 are strains of *E. coli*; all other strains are *S. typhimurium*. Conjugations and transductions were performed as described in the Methods section and antibiotic selections were as indicated using medium B. The glucose utilization phenotype was determined with BTB and TZ plates. (It was necessary to move the *pgi*::Tn10 through a second *mut* strain (HR150) before it could successfully be moved into the HR104 background. The most probable reason for this may have been to reduce flanking mismatch regions during recombination prior to transfer to a *mut*$^+$ background.) Antibiotic concentrations in medium B were as follows: streptomycin, 100ug/ml; tetracycline, 25ug/ml and kanamycin, 50ug/ml.

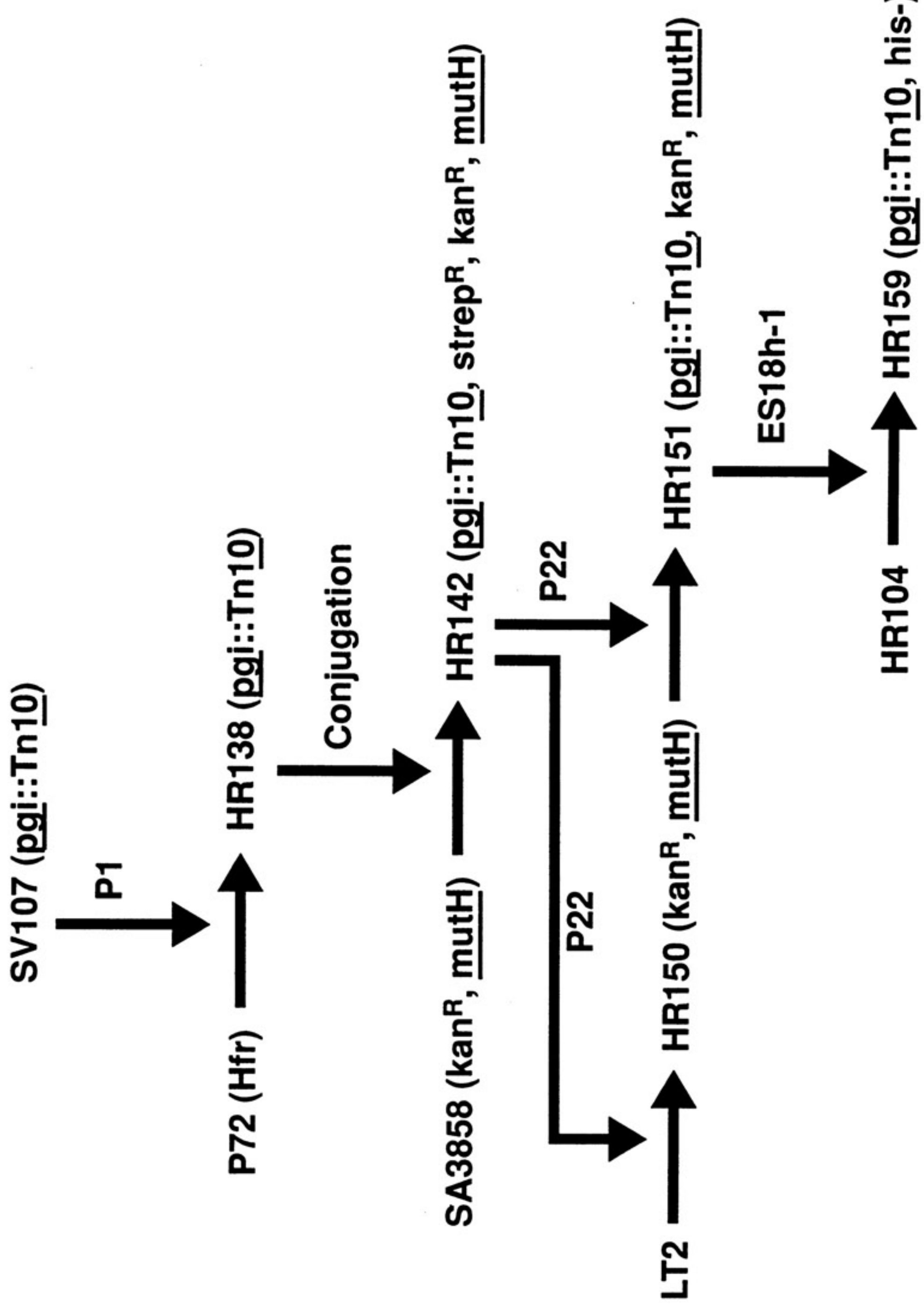
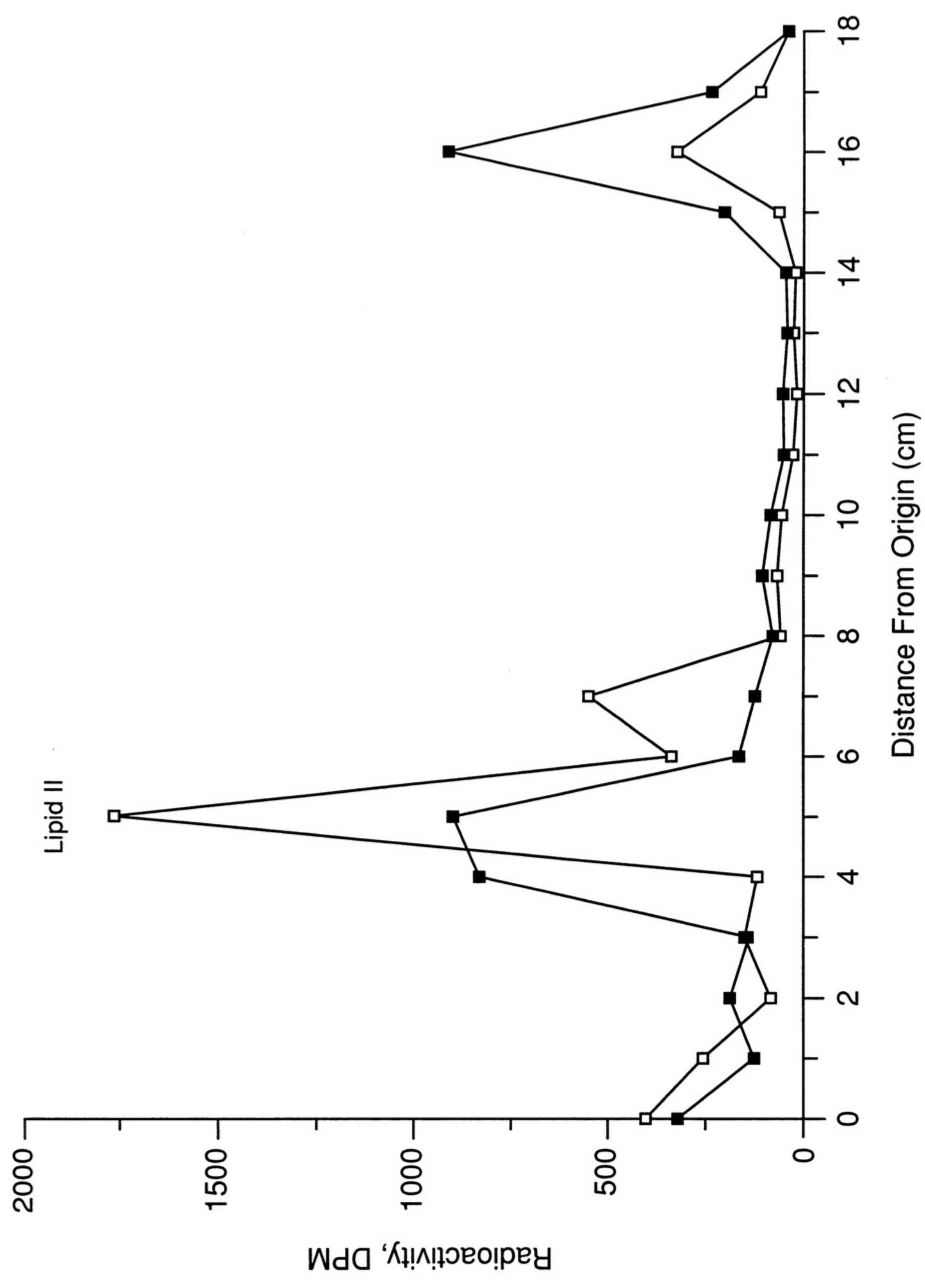

SV107 (pgi::Tn10)
P1
P72 (Hfr)
HR138 (pgi::Tn10)
Conjugation
SA3858 (kanR, mutH)
HR142 (pgi::Tn10, strepR, kanR, mutH)
P22
P22
LT2
HR150 (kanR, mutH)
HR151 (pgi::Tn10, kanR, mutH)
ES18h-1
HR104
HR159 (pgi::Tn10, his-)

Figure 19. Accumulation of Lipid II in Strains HR104 and HR159. Strains HR104 (ECA-trace) and HR159 (ECA-trace, *pgi*::Tn10) were grown at 37°C in medium C with 2% glycerol as the sole carbon source. The medium was also supplemented with histidine (0.1 mM final concentration). Mid-log phase cells were labeled with [^{3}H]GlcNAc for 30 minutes and then harvested. The cells were next carried through the extraction procedure for the isolation of Lipid II, and the extracts were analyzed by SG81 paper-chromatography using solvent system B as described in the Methods section. Symbols: □, HR159 (*pgi*::Tn10); ■, HR104. The lipid II standard was partially purified by DEAE-chromatography as detailed in the Methods section, and it was applied to a separate lane on the SG81 chromatogram.



Lipid II
Radioactivity, DPM
0
500
1000
1500
2000
Distance From Origin (cm)
0
2
4
6
8
10
12
14
16
18

Figure 20. Effect of Exogenously Supplied Glucose on the Ability of Strain HR159 to Synthesize Trace ECA. Strains HR104 (ECA-trace) and HR159 (ECA-trace, *pgi*::Tn10) were grown overnight at 37°C in medium C with 2% glycerol as the sole carbon source. The medium was also supplemented with histidine (0.1 mM final concentration). Duplicate cultures of each strain were also grown in medium C containing 0.2% glucose as the sole carbon source. The cells were then harvested and analyzed for the presence of ECA by Western blot analysis as described in the Methods section. Lane A, HR104 (Minimal media); Lane B, HR104 (Minimal media with glucose); Lane C, HR159 (Minimal media); Lane D, HR159 (Minimal media with glucose); Lane E, ECA standard (PR122).

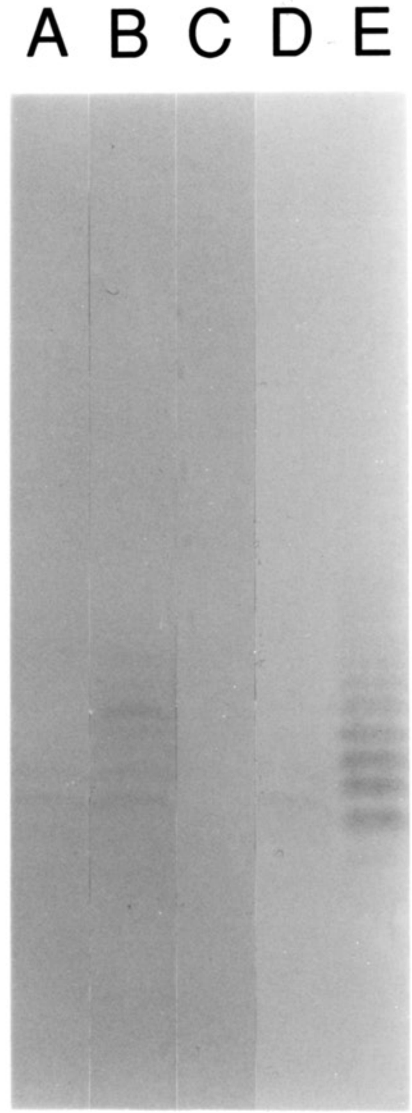
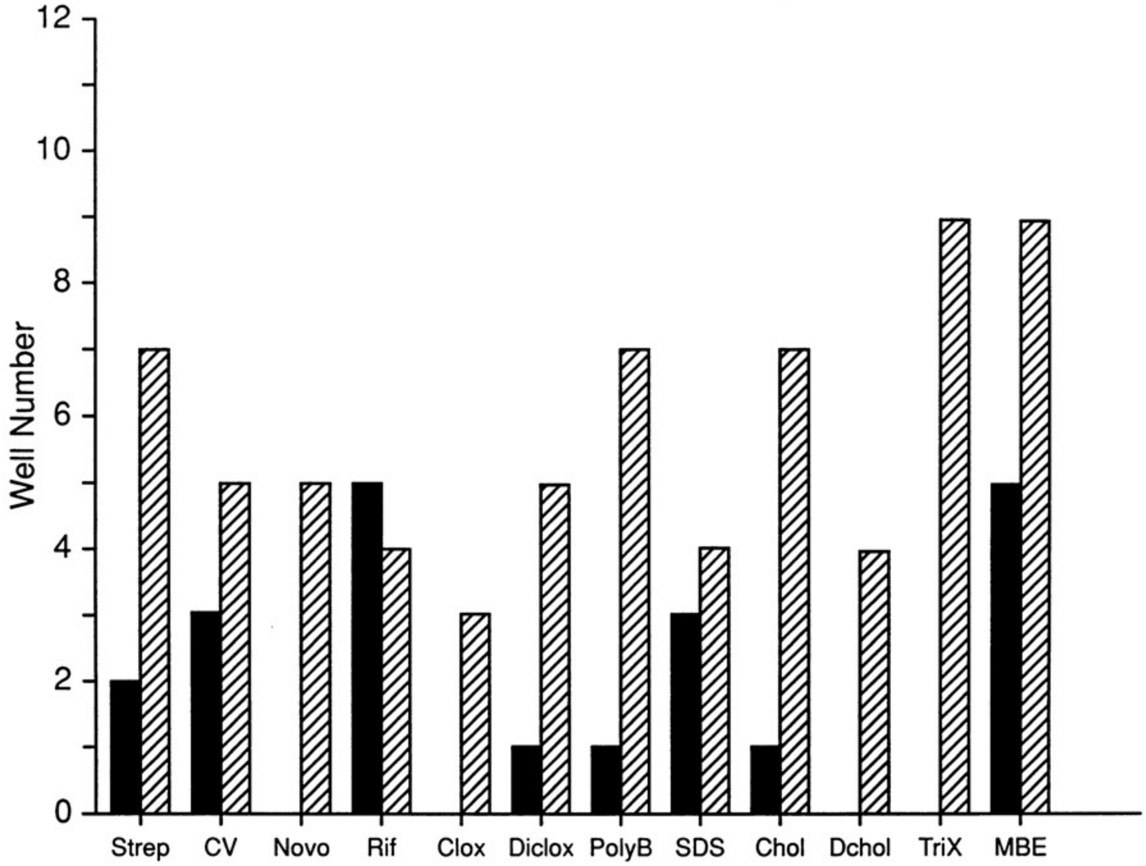

A B C D E

Figure 21. Effect of Media Composition on the Sensitivity of Strain HR104 to Various Agents. Strain HR104 (ECA-trace) was tested for sensitivity to various agents in medium C (minimal media) containing 2% glycerol as the sole carbon source. The medium was also supplemented with histidine (0.1 mM final concentration). Antibiotic and hydrophobic agent stock solutions were diluted in medium C and distributed to 96-well microtiter plates in serial 2-fold dilutions as detailed in the Methods section. Strain HR104 was grown overnight in medium C (supplemented as indicated above) and prepared for addition to the 96-well plates as described in the Methods. Results obtained using rich media were derived from Tables 5-7 where medium A supplemented with 0.2% glucose was used. The data are expressed as the last well in which no growth was observed. The concentration ranges of agents were identical for both media. Agent abbreviations are as follows: Strep, streptomycin; CV, crystal violet; Novo, novobiocin; Rif, rifamycin; Clox, cloxacillin; Diclox, dicloxacillin; PolyB, polymyxin B; SDS, sodium dodecylsulfate; Chol, sodium cholate; Dchol, sodium deoxycholate; TriX, triton X100; MBE, methylbenzylethonium chloride; Amp, ampicillin; Penn, pennicillin G; Tet, tetracycline; Otet, oxytetracycline; Carb, carbenicillin; Ceph, cephalosporin C; ActD, actinomycin D; Kan, kanamycin; Ctet, chlorotetracycline; Van, vancomycin; MalG, malachite green; Eryth, erythromycin; Bacit, bacitracin. ▨ Rich Media, ■ Minimal Media.


12
10
8
6
4
2
0
Well Number
Strep
CV
Novo
Rif
Clox
Diclox
PolyB
SDS
Chol
Dchol
TriX
MBE

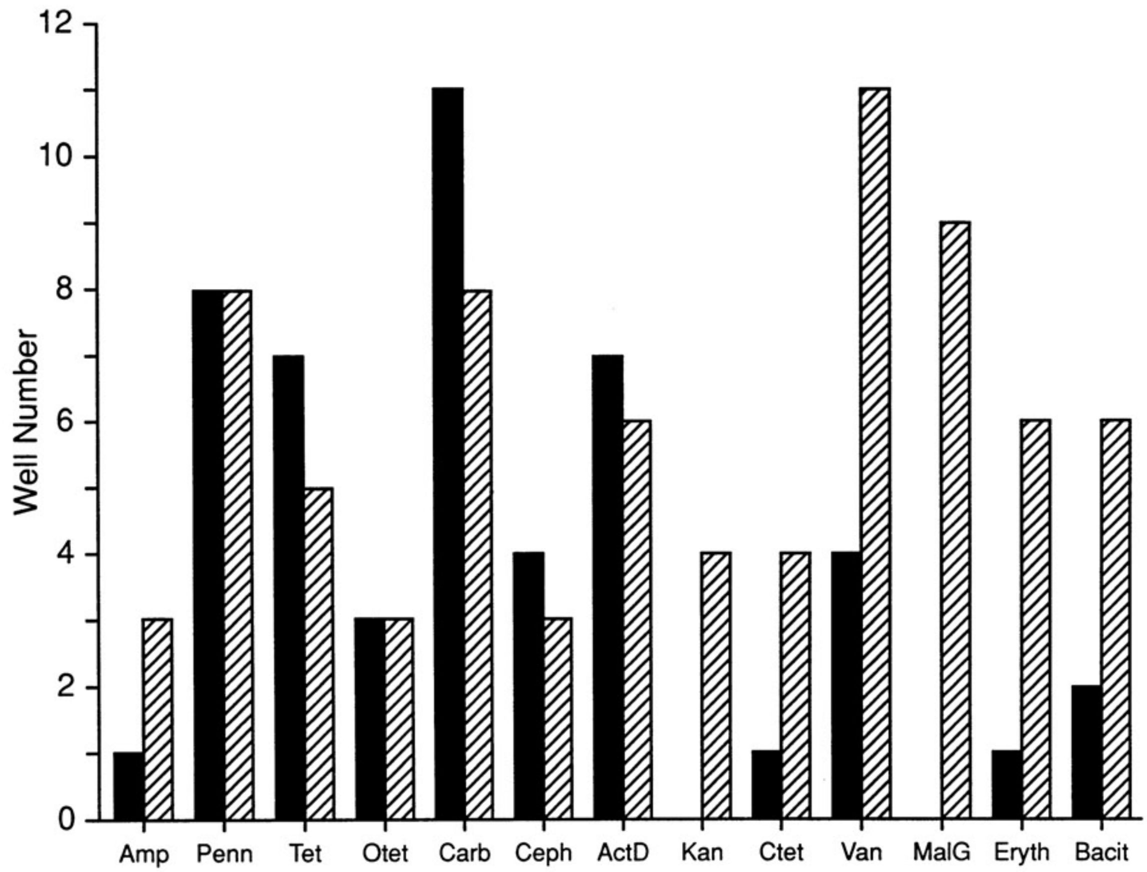
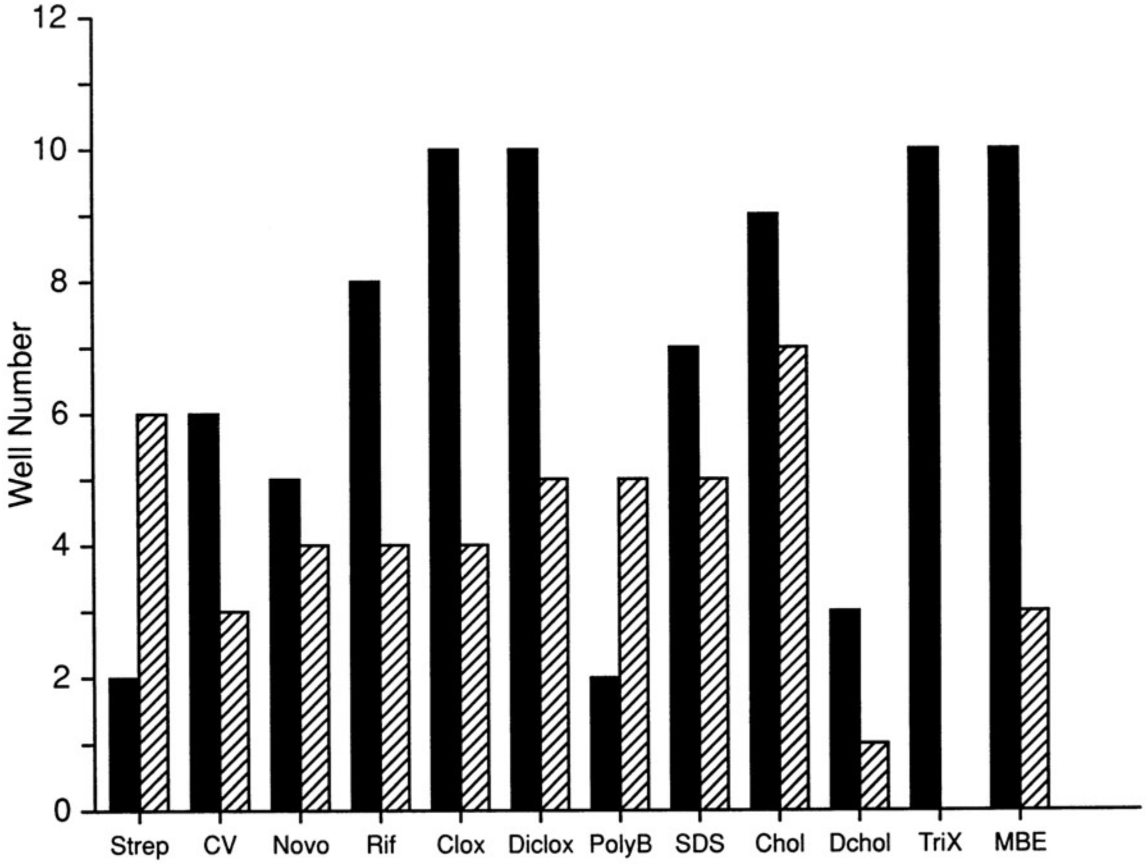

12
10
8
6
4
2
0
Well Number
Amp
Penn
Tet
Otet
Carb
Ceph
ActD
Kan
Ctet
Van
MalG
Eryth
Bacit

Figure 22. Effect of Media Composition on the Sensitivity of Strain HR159 to Various Agents. Strain HR159 (ECA-trace, *pgi*) was tested for sensitivity to various agents in medium C (minimal media) supplemented with 2% glycerol as the sole carbon source. The medium was also supplemented with histidine (0.1 mM final concentration). Other details are as in the legend to Figure 21. Strain HR159 was grown overnight in medium C (supplemented as indicated above) and prepared for addition to the 98-well plates as described in the Methods. Rich media results were derived from Tables 5-7. ▨ Rich Media, ■ Minimal Media.


12
10
8
6
4
2
0
Well Number
Strep
CV
Novo
Rif
Clox
Diclox
PolyB
SDS
Chol
Dchol
TriX
MBE

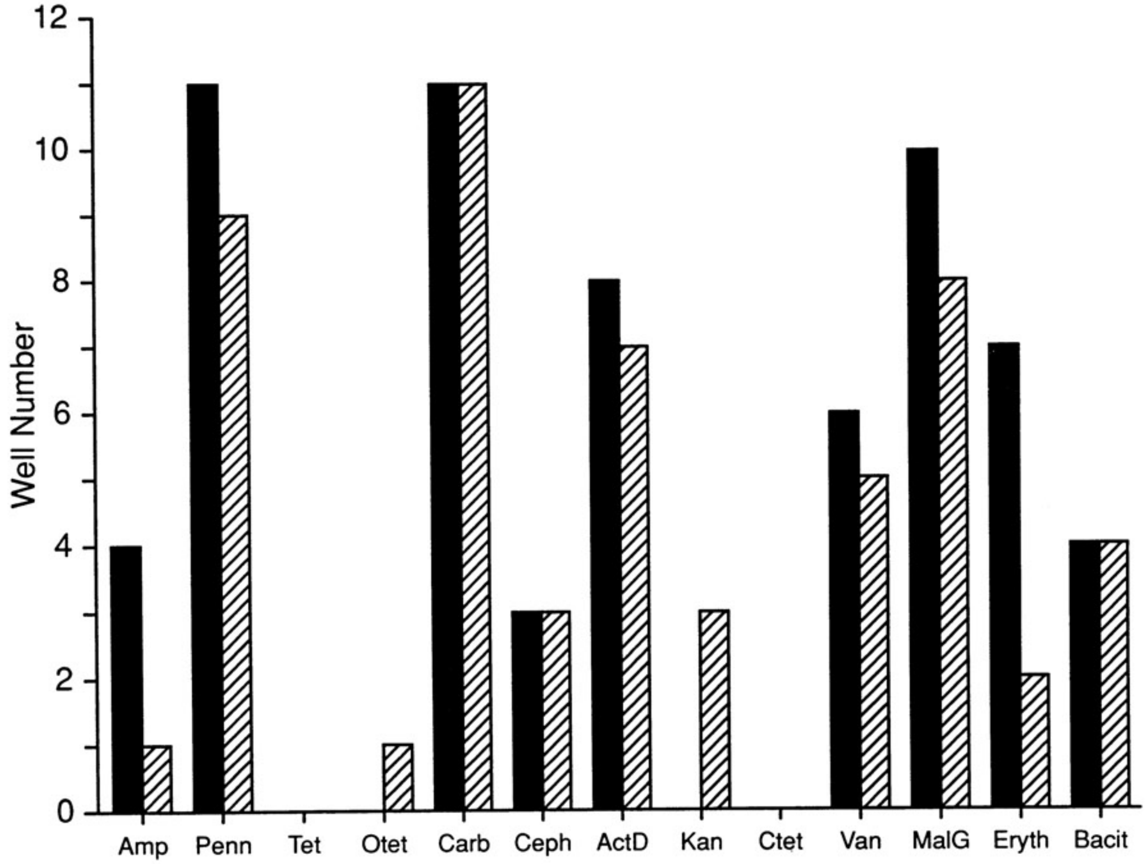
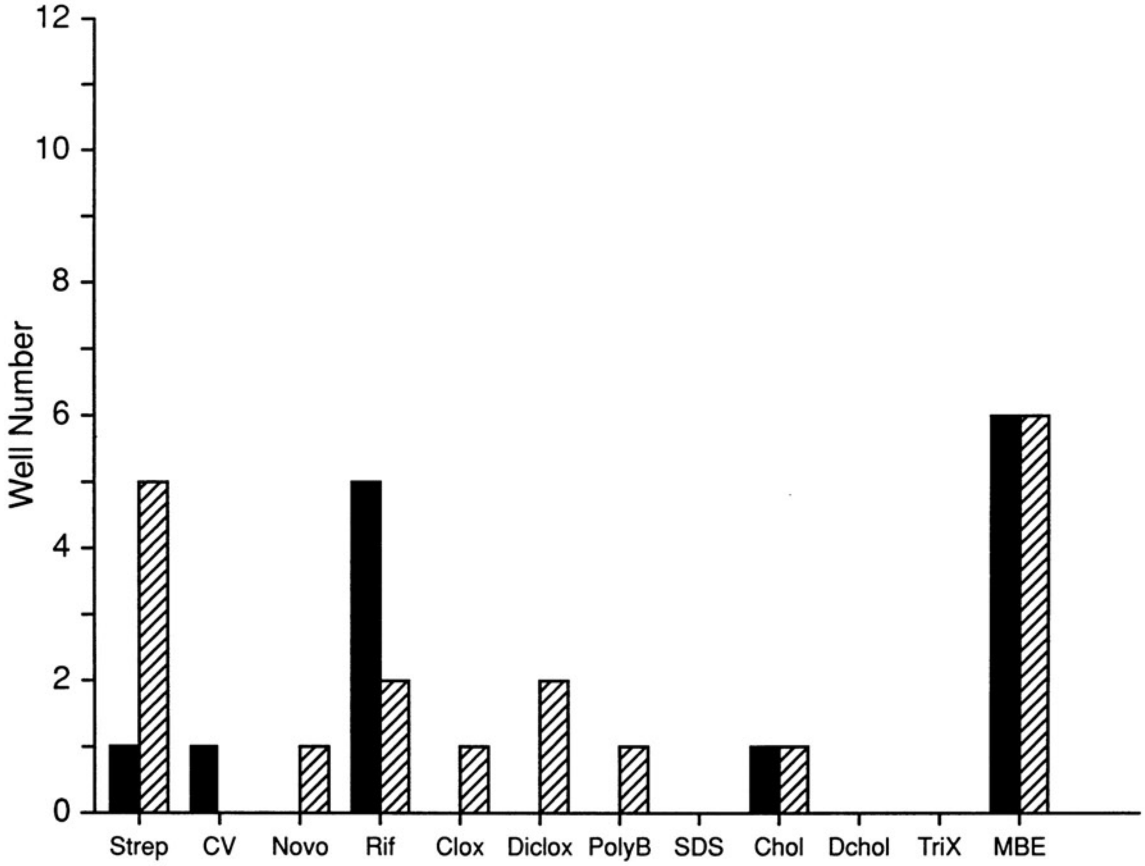
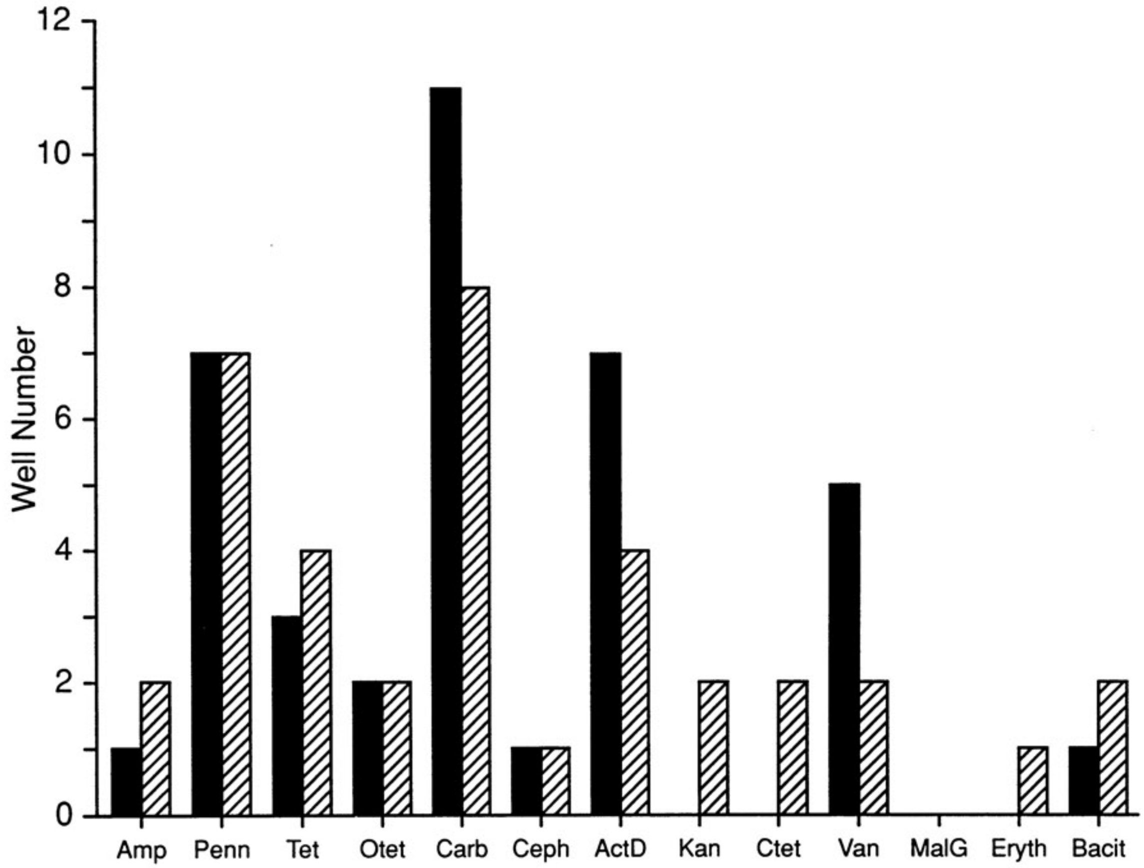

12
10
8
6
4
2
0
Well Number
Amp
Penn
Tet
Otet
Carb
Ceph
ActD
Kan
Ctet
Van
MalG
Eryth
Bacit

Figure 23. Effect of Media Composition on the Sensitivity of Strain LT2 to Various Agents. Strain LT2 (wild type) was tested for sensitivity to various agents in medium C (minimal media) supplemented with 2% glycerol as the sole carbon source. Other details are as in the legend to Figure 21. Strain LT2 was grown overnight in medium C (supplemented as indicated above) and prepared for addition to the 98-well plates as described in the Methods. Rich media results were derived from Tables 5-7. ▨ Rich Media, ■ Minimal Media.

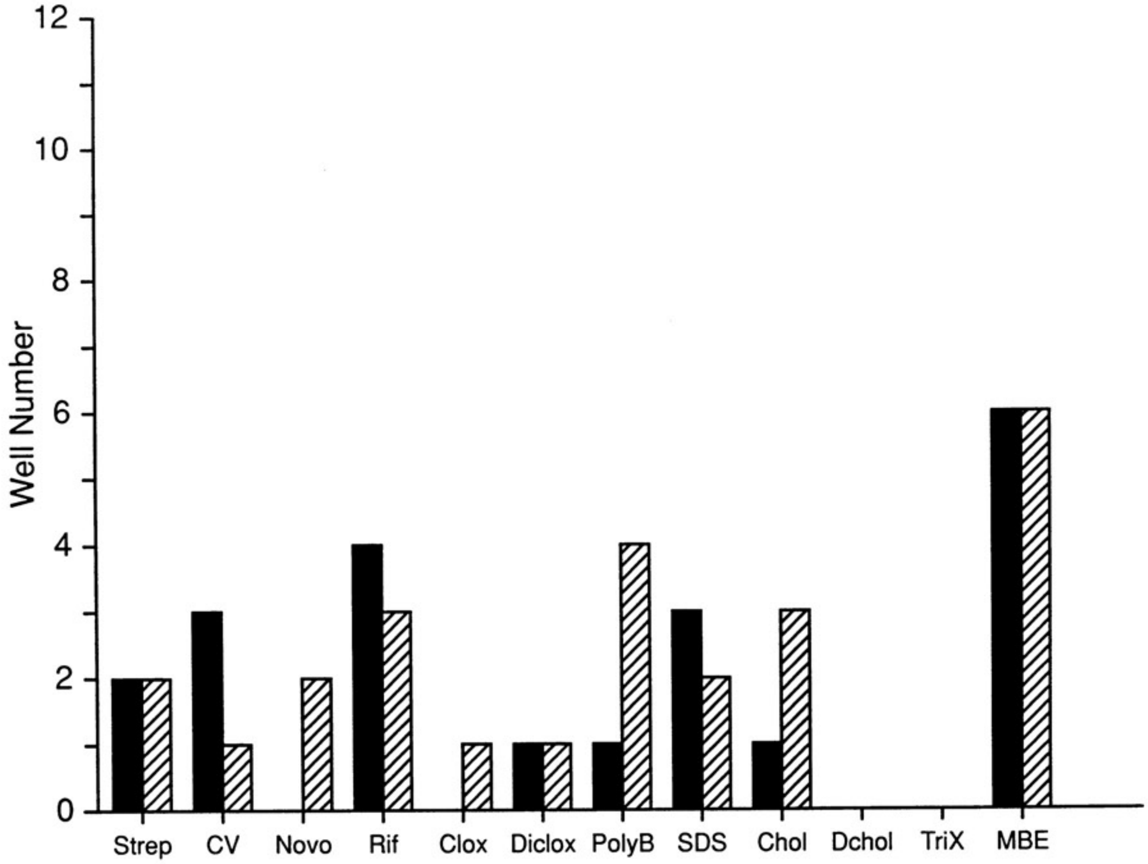


12
10
8
6
4
2
0
Well Number
Strep
CV
Novo
Rif
Clox
Diclox
PolyB
SDS
Chol
Dchol
TriX
MBE

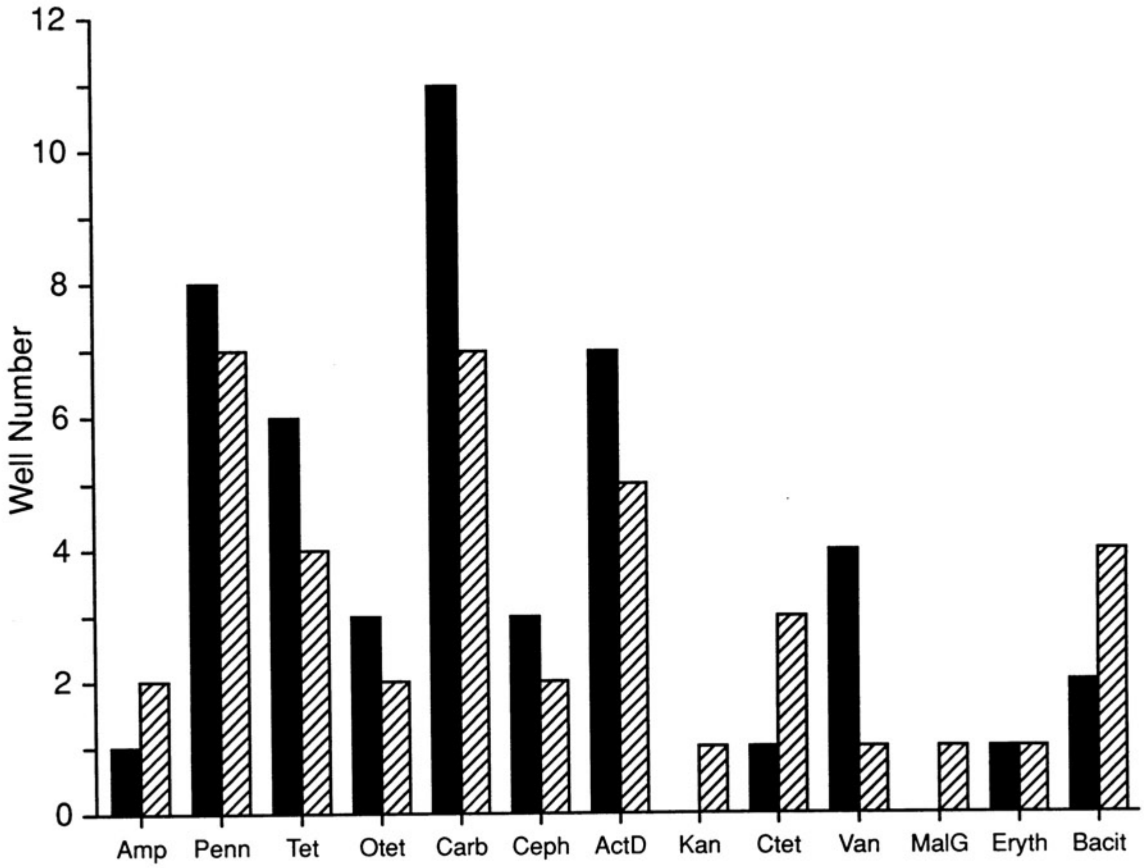


12
10
8
6
4
2
0
Well Number
Amp
Penn
Tet
Otet
Carb
Ceph
ActD
Kan
Ctet
Van
MalG
Eryth
Bacit

Figure 24. Effect of Media Composition on the Sensitivity of Strain SH5150 to Various Agents. Strain SH5150 (ECA-) was tested for sensitivity to various agents in medium C (minimal media) supplemented with 2% glycerol as the sole carbon source. The medium was also supplemented with the following amino acids to give final concentrations as indicated: histidine (0.1mM), isoleucine (0.3mM), valine (0.3mM), and methionine (0.3mM). Other details are as in the legend to Figure 21. Strain HR159 was grown overnight in medium C (supplemented as indicated above) and prepared for addition to the 98-well plates as described in the Methods. Rich media results were derived from Tables 5-7. ▨ Rich Media, ■ Minimal Media.

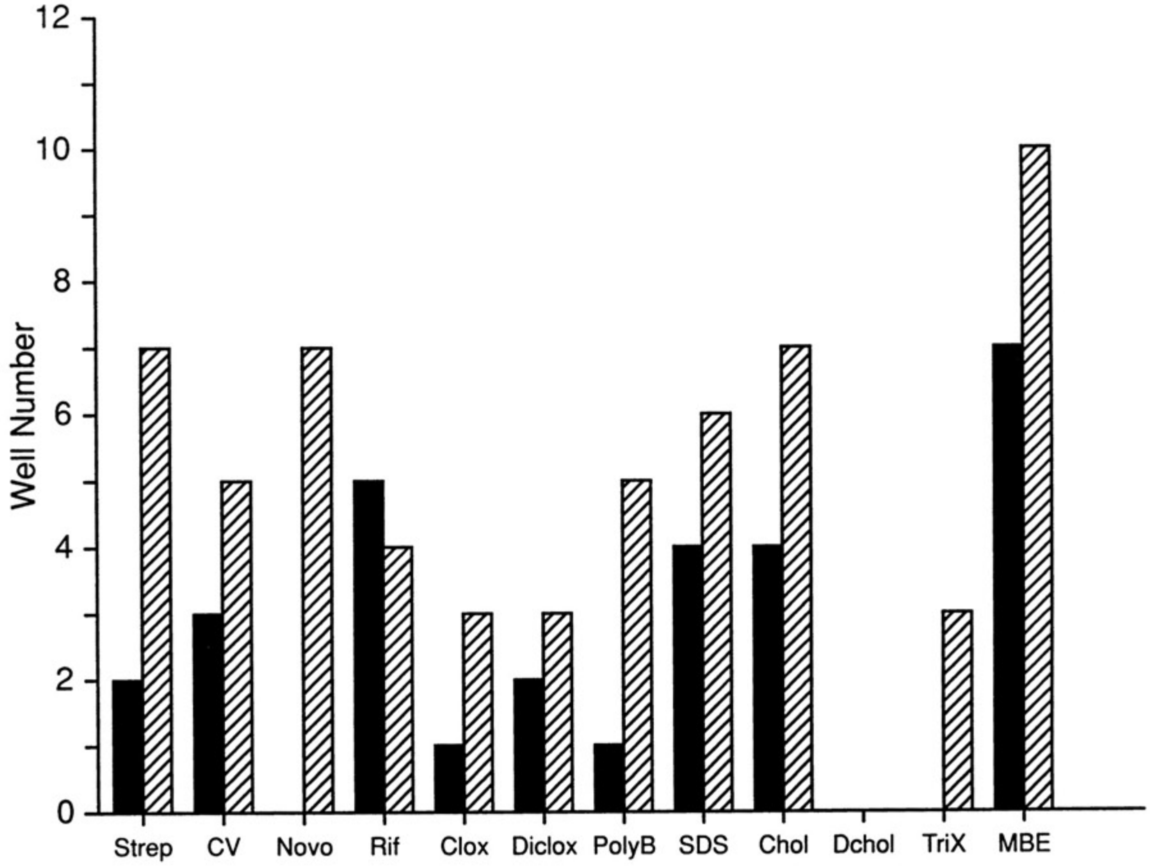


Well Number
0
2
4
6
8
10
12
Strep
CV
Novo
Rif
Clox
Diclox
PolyB
SDS
Chol
Dchol
TriX
MBE



Well Number
0
2
4
6
8
10
12
Amp
Penn
Tet
Otet
Carb
Ceph
ActD
Kan
Ctet
Van
MalG
Eryth
Bacit

Figure 25. Effect of Media Composition on the Sensitivity of Strain SL3769 to Various Agents. Strain SL3769 (Rd1-LPS) was tested for sensitivity to various agents in medium C (minimal media) supplemented with 2% glycerol as the sole carbon source. Other details are as in the legend to Figure 21. Strain SL3769 was grown overnight in medium C (supplemented as indicated above) and prepared for addition to the 98-well plates as described in the Methods. Rich media results were derived from Tables 5-7. ▨ Rich Media, ■ Minimal Media.


12
10
8
6
4
2
0
Well Number
Strep
CV
Novo
Rif
Clox
Diclox
PolyB
SDS
Chol
Dchol
TriX
MBE

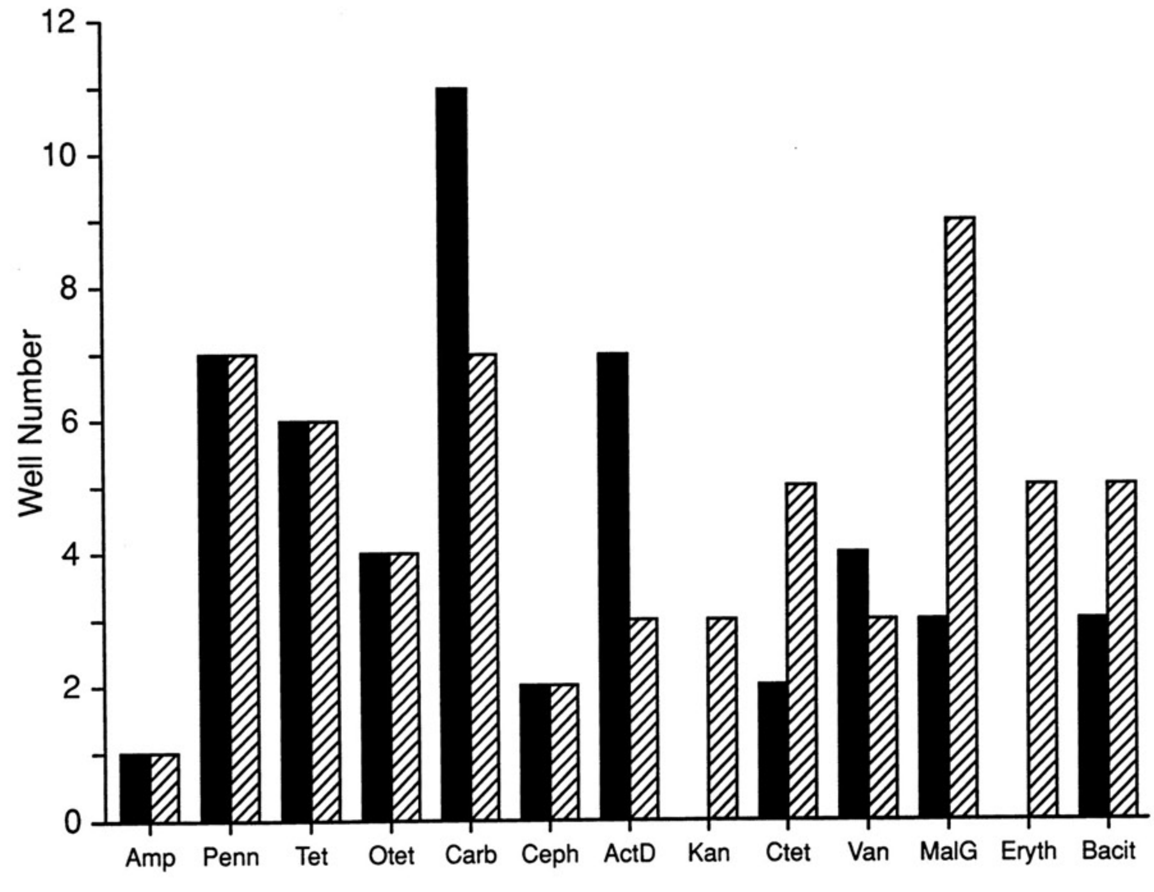
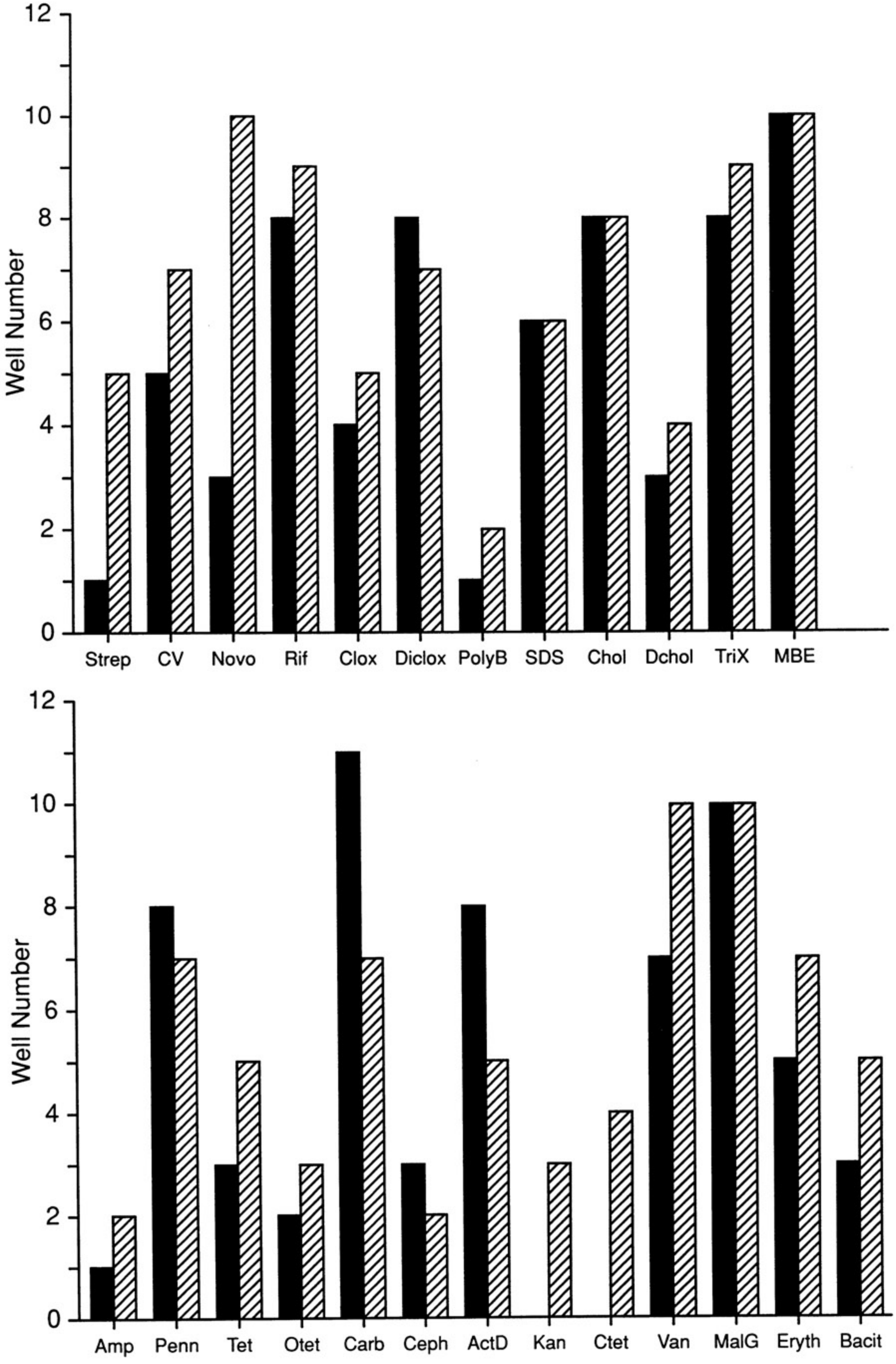

12
10
8
6
4
2
0
Well Number
Amp
Penn
Tet
Otet
Carb
Ceph
ActD
Kan
Ctet
Van
MalG
Eryth
Bacit

Figure 26. Effect of Media Composition on the Sensitivity of Strain G30A to Various Agents. Strain G30A (Re-LPS) was tested for sensitivity to various agents in medium C (minimal media) supplemented with 2% glycerol as the sole carbon source. Other details are as in the legend to Figure 21. Strain G30A was grown overnight in medium C (supplemented as indicated above) and prepared for addition to the 98-well plates as described in the Methods. Rich media results were derived from Tables 5-7. ▨ Rich Media, ■ Minimal Media.



Well Number
0
2
4
6
8
10
12
Strep
CV
Novo
Rif
Clox
Diclox
PolyB
SDS
Chol
Dchol
TriX
MBE
Well Number
Amp
Penn
Tet
Otet
Carb
Ceph
ActD
Kan
Ctet
Van
MalG
Eryth
Bacit

Therefore, attempts were made to construct a derivative of strain HR104 that was unable to synthesize "trace-ECA" but was still able to accumulate lipid II without altering the structure of LPS. Mutations in the genes encoding either the transaminase or the transacetylase enzymes that are responsible for converting TDP-4-keto-6-deoxy-D-glucose to TDP-FucNAc (Figure 3) or the TDP-Fuc4NAc transferase would result in this phenotype. Mutations in these genes have not been identified in *S. typhimurium*, but they have recently been characterized in *E. coli* (Meier-Dieter et al., 1990). Accordingly, attempts were made to construct a derivative of strain HR104 possessing a defective *rffT* gene from an *E. coli* mutant. The *S. typhimurium* mismatch-repair mutant, SA3858 (Rayssiguier et al., 1989) was again used for this construction. A P1 lysate of *E. coli* strain 21731 (*rffT*, *zie*2::Tn<u>10</u>) was transduced into strain SA3858 and transductants were selected on medium B plates containing 25ug/ml tetracycline. Strain SA3858 also has a mutation in *ilv* which proved fortuitous since it provided an additional screening step for the transductants. The transductants were transferred to medium C plates containing 1% glucose, 0.1mM tryptophan, 0.3mM methionine and 100ug/ml streptomycin in order to screen for *ilv*$^{+}$ transconjugates. The co-transduction frequency of tetracycline resistance and *ilv* was determined to be 80% and was in close agreement with previous results obtained in experiments using *E. coli* (Meier and Mayer, 1985). The *ilv*-plus transductants were further screened by the colony immunoblot assay for the presence of ECA. One ECA-minus transductant, strain HR210, was identified and characterized in further detail. This strain was found to accumulate lipid II whereas lipid II was not detected in the parent strain SA3858 (Figure 27). Western blot analysis verified that strain HR210 was ECA-minus (Figure 28). However, strain HR210 was SDS sensitive while

Figure 27. Accumulation of Lipid II in Strains HR210 and SA3858. Strains HR210 (*rffT*) and SA3852 (*rffT* + parent strain) were grown at 37°C in medium A supplemented with 0.2% glucose. Mid-log phase cells were labeled with [^{3}H]GlcNAc for 30 minutes and then harvested by centrifugation. The cells were next carried through the extraction procedure for the isolation of Lipid II, and the extracts were analyzed by SG81 paper-chromatography using solvent system B as described in the Methods section. Symbols: □ SA3858, ■ HR210. Lipid I and Lipid II standards were partially purified by DEAE-chromatography as detailed in the Methods section and applied to separate lanes on the SG81 chromatogram.

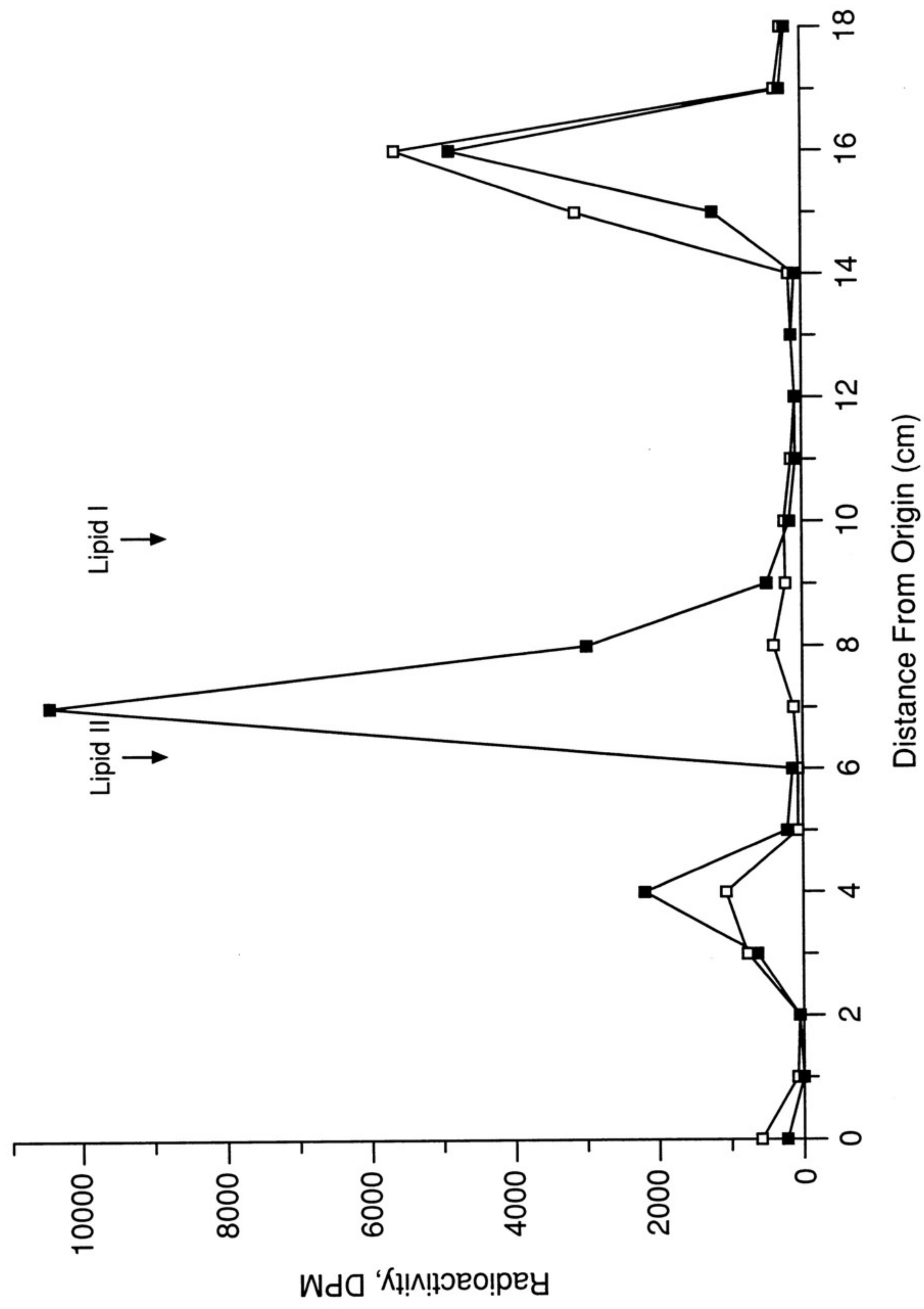

Radioactivity, DPM
10000
8000
6000
4000
2000
0
Distance From Origin (cm)
0
2
4
6
8
10
12
14
16
18
Lipid II
Lipid I

Figure 28. Effect of the *rffT* Mutation on ECA as Determined by Western Blot Analysis. Strains SA3858 (ECA+) and HR210 (*rffT*) were grown overnight in medium A supplemented with 0.2% glucose at 37°C. The cells were then harvested and analyzed for the presence of ECA by Western blot analysis as described in the Methods section. Lane A, HR210; Lane B, SA3858.


A
B

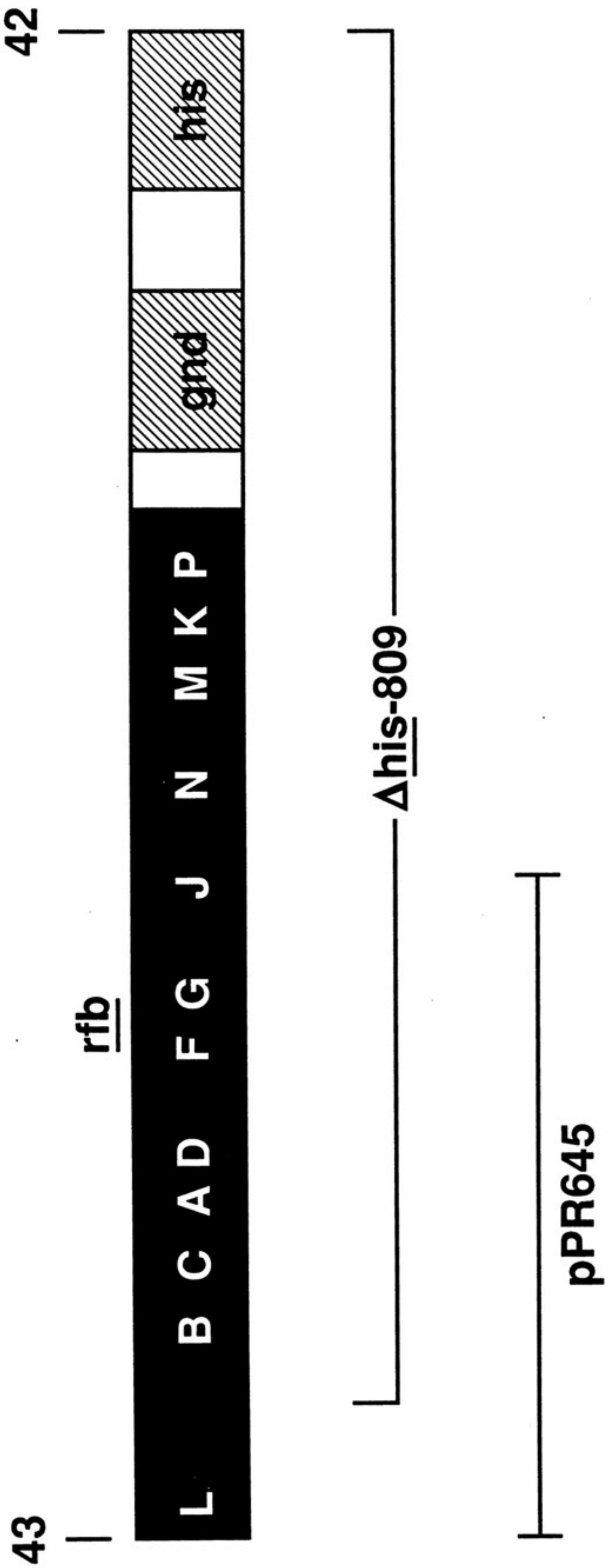
SA3858 was resistant to SDS. Numerous attempts to move the *rffT* mutation into the ECA-trace strain, HR104 were unsuccessful as we were not able to obtain any Tet^r transductants.

Construction of an *rfbA+ rfbB+* Derivative of an "ECA-trace" Strain.

The ECA-trace strains, in addition to missing much of the *rfb* locus, are also missing part of the *his* operon as well as all intervening genes due to the *his-rfb* deletion (Figure 29) (Nikaido et al., 1967). Analysis of *S. typhimurium rfb* deletion mutants indicated that only the *rfbA* and *rfbB* genes are involved in ECA biosynthesis and the sensitivity of these mutants to hydrophobic agents (Mäkelä et al., 1976). However, no *rfb* point mutation has been identified that results in the ECA-trace phenotype. Accordingly, experiments were conducted in order to evaluate the role, if any, of other genes in the *his-rfb* region in the ECA-trace phenotype. The *rfb* region has recently been cloned, physically mapped and sequenced in the laboratory of Dr. Peter Reeves (Brahmbhatt et al., 1986; Brahmbhatt et al., 1988; Jiang et al., 1991). Thus, we obtained a plasmid (plasmid pPR645) from Dr. P. R. Reeves that contains the *rfbA* and *rfbB* genes and, with the exception of several flanking *rfb* genes (*rfbC, rfbD, rfbF, rfbG*), does not complement mutations in other genes in the *his-rfb* region (Figure 29).

Plasmid pPR645 contains an 11Kb EcoR1 fragment of the *rfb* region cloned into the EcoR1 site of vector pKO1 (Brahmbhatt et al., 1988; McKenney et al., 1981). Plasmids pPR645 and pKO1 were isolated from strains P3778 and N100, respectively, and aliquots were restriction digested with EcoR1 and characterized by electrophoresis on 1% agarose gels. Plasmid pKO1 is 3.9Kb and pPR645 yielded 11Kb and 3.9Kb fragments. P3778 and N100 are

Figure 29. Map of the *his-rfb* Region of the *Salmonella typhimurium* Chromosome. The map shows the relative location of genes in the *his-rfb* region, located between 42 and 43 minutes on the *S. typhimurium* chromosome. Abbreviations are *his*, histidine biosynthesis gene region; *gnd*, gluconate-6-phosphate dehydrogenase structural gene; and *rfb*, O-antigen biosynthesis. The letters represent various known structural genes for nucleotide sugar synthases and glycosyl transferases involved in O-antigen synthesis. The *rfbA* and *rfbB* genes are the structural genes for TDP-glucose pyrophosphorylase and TDP-glucose oxidoreductase, respectively. The *rfbA* and *rfbB* gene products are also involved in ECA biosynthesis. Also indicated is the his809 deletion mutation found in strain SH5150 and all ECA-trace strains derived from this strain. Plasmid pPR645 contains the indicated fragment of the *rfb* region. Note that the *his*809 deletion includes the *rfbA* and *B* genes and that these genes are within the pPR645 fragment.


42
43
his
gnd
L B C A D F G J N M K P
rfb
Δhis-809
pPR645

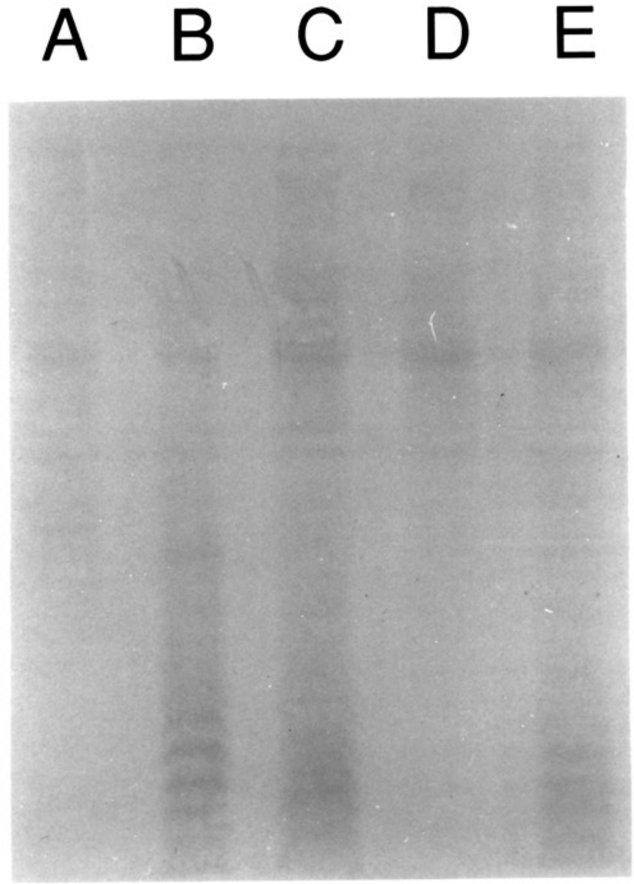
E. coli strains, therefore in order to move the plasmids into *S. typhimurium* they had to be passaged through a restriction-minus and modification-plus strain. Both plasmids were transformed into *S. typhimurium* strain LB5010(r^-m^+) (Bullas and Ryu, 1983). The plasmids were reisolated from strain LB5010 transformants and their restriction fragment pattern was verified following digestion with EcoR1. Although we were unable to transform the *Salmonella* modified plasmids isolated from LB5010 into the ECA-trace and control strains using the competent cell procedure, these transformations were accomplished by electroporation. Transformants of strains SH5150 and HR197 were selected by resistance to ampicillin (100ug/ml) on medium B plates and screened for parent strain auxotrophies on medium C plates. The plasmids were then reisolated and confirmed by EcoR1 restriction fragment analysis. Strains HR206 and HR209 are pKO1 and pPR645 transformants of the ECA-minus strain SH5150 (*rff*, Δ*rfb*), respectively. Strains HR207 and HR208 are the pKO1 and pPR645 transformants of the ECA-trace strain HR197 (Δ*his-rfb*), respectively. The TDP-Glucose oxidoreductase activity of the transformants was assayed to determine the presence and expression of pPR645, and these analyses revealed that only strains HR208 and HR209 showed activity (Table 9). The transformants were then characterized for ECA, the accumulation of lipid-linked ECA intermediates, and SDS sensitivity. Western blot analysis showed that the presence of pPR645 in the ECA-trace strain (HR208) complemented the defect in ECA synthesis; however, none of the other transformants showed an altered ECA phenotype from that of their respective parental strain (Figure 30). SG81 chromatography of chloroform/methanol (3:2, v/v) extracts from the transformants also showed a reduction of lipid II

Table 9. Specific Activities of TDP-Glucose Oxido-Reductase

Strain	Relavant Genotype	Specific activity *
PR122	(*rfb*$^{+}$)	7.2
SH5150	(Δ*rfb*, *rff*)	0
HR197	(Δ*rfb*)	1.4
HR206	(Δ*rfb*, *rff*, pKO1)	0.2
HR209	(Δ*rfb*, *rff*, pPR645)	22.6
HR207	(Δ*rfb*, pKO1)	1.5
HR208	(Δ*rfb*, pPR645)	24.7

* Specific activity is expressed as nmole/min/mg protein.
Enzymes were assayed as described in the Methods. Protein was assayed by the BCA method.

Figure 30. Western Blot Analysis of ECA-trace strains transformed with pPR645. Strains were grown overnight in medium A supplemented with 0.2% glucose at 37°C. For those strains containing plasmids pKO1 or pPR645, ampicillin was also added to give a final concentration of 100ug/ml. The cells were then harvested and analyzed for the presence of ECA by Western blot analysis as described in the Methods section. Lane A, HR209 (ECA^-, pPR645); Lane B, HR208 (ECA-trace, pPR645); Lane C, HR207 (ECA-trace, pKO1); Lane D, HR206 (ECA^-, pKO1); Lane E, HR197 (ECA-trace). Note that lanes A, C, D, and E have three times the material (30ul) loaded onto the gel as lane B (10ul).


A B C D E

accumulation in HR208 (Figure 31). The SDS sensitivity of strain HR208 was also altered and this strain was no longer SDS-sensitive (Figure 32).

LPS Composition of ECA-trace Strains

LPS was isolated from strains SH5150 and HR104 as described in the Methods. The yields of LPS from 50-liter cultures were 298mg/20gm cell dry weight (1.49%) for strain SH5150 and 301mg/15.6gm cell dry weight (1.93%) for strain HR104. The core oligosaccharides were released by mild acid hydrolysis and isolated for analysis as described in the Methods.

Released oligosaccharides were analyzed using the Dionex BioLC system. Eluant 1 was 300mM NaOH, eluant 2 was 500mM sodium acetate in 200mM NaOH, eluant 3 was water and eluant 4 was 500mM sodium acetate. The starting conditions were eluant 1 at 53%, eluant 2 at 20% and eluant 3 at 27% and a sodium acetate gradient was produced by a linear increase of eluant 4 from 0% to 100% over a 90 minute period. (The percentages represent the amount of each eluant that is present in the elution stream.) The flow rate was 1.0ml/minute. Samples were injected using a 25ul sample loop. To enhance detection at the PAD, 100mM NaOH was added post column at a flow rate of 0.5ml/minute. The results are summarized in Figures 33 and 34 for strains SH5150 and HR104, respectively. Although the pattern of separated oligosaccharides is similar, there appears to be significant quantitative differences. The LPS-oligosaccharide pattern for the ECA-trace strain HR104 resembles the separation pattern of the mixture of LPS chemotype standards (Figure 35). A significant amount of material from the LPS of strain HR104 elutes in the early regions of the chromatogram (0-20 minutes), and it appears to correspond to oligosaccharides released from Rc,

Figure 31. Accumulation of Lipid II in pPR645 Transformants. Strains were grown at 37°C in medium A supplemented with 0.2% glucose. For those strains containing plasmids pKO1 or pPR645, ampicillin was also added to give a final concentration of 100ug/ml. Mid-log phase cells were labeled with [^{3}H]GlcNAc for 30 minutes and then harvested by centrifugation. The cells were next carried through the extraction procedure for the isolation of Lipid II, and the extracts were analyzed by SG81 paper-chromatography using solvent system B as described in the Methods section. Symbols: □, HR206 (ECA-, pKO1), ■, HR209 (ECA-, pPR645), ○, HR207 (ECA-trace, pKO1), ●, HR208 (ECA-trace, pPR645). Lipid I and Lipid II standards were partially purified by DEAE-chromatography as detailed in the Methods section and applied to separate lanes on the SG81 chromatogram.

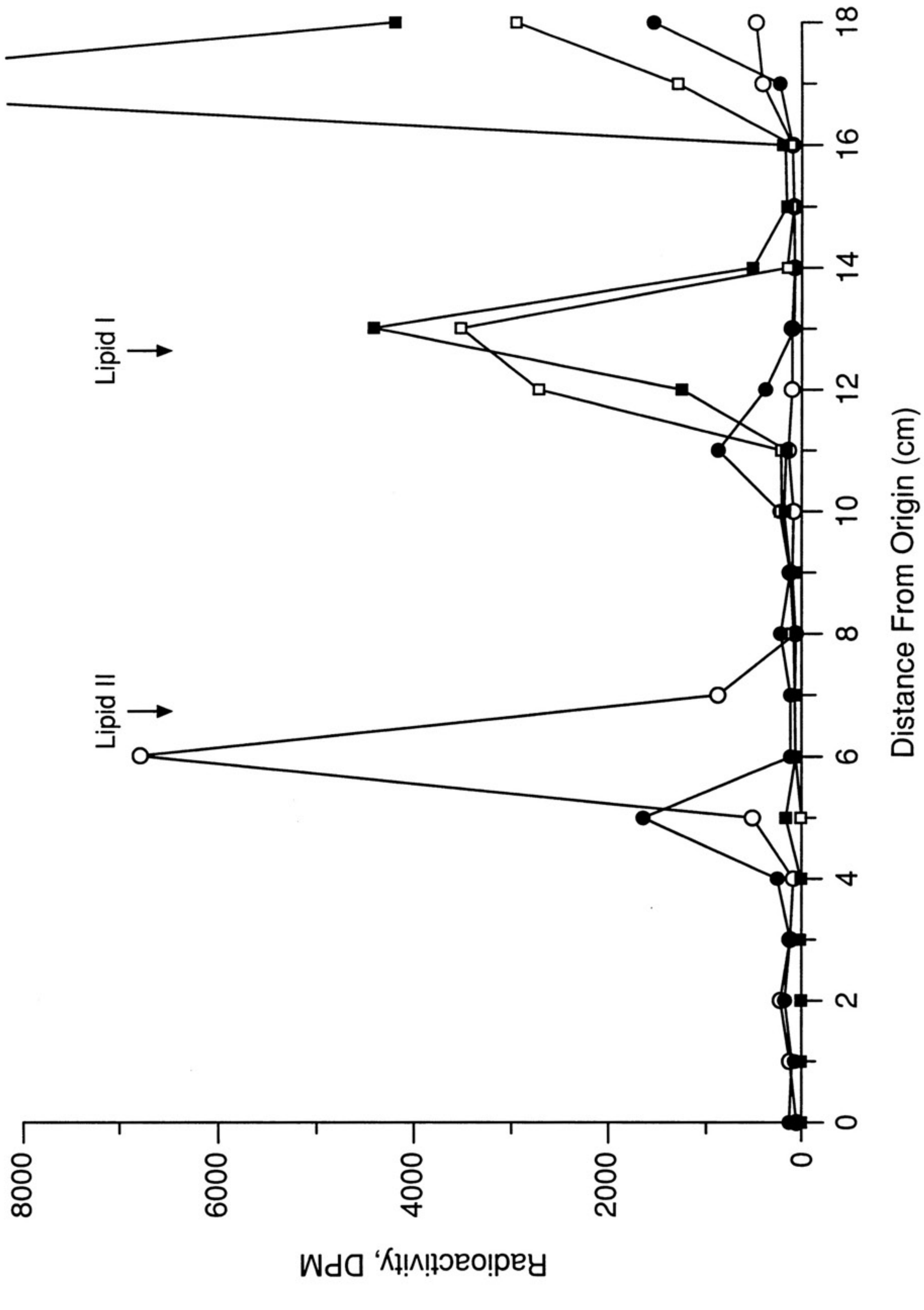

Radioactivity, DPM
8000
6000
4000
2000
0
0
2
4
6
8
10
12
14
16
18
Distance From Origin (cm)
Lipid II
Lipid I

Figure 32. SDS Sensitivities of pPR645 Transformants. Strains transformed with pPR645 or pKO1 were streaked across medium A plates. A sterile disk saturated with 1% SDS was placed on the "lawn" surface and the plates were then incubated overnight at 37°C. Transformant medium A plates also contained ampicillin at a final concentration of 100ug/ml. A, SH5150 (ECA-); B, HR197 (ECA-trace); C, HR206 (ECA-, pKO1); D, HR209 (ECA-, pPR645); E, HR208 (ECA-trace, pPR645); F, HR207 (ECA-trace, pKO1).


F
E
B
A
D
C

Figure 33. Analysis of Oligosaccharides Released by Mild Acid from LPS Isolated from SH5150. LPS isolated from strain SH5150 (ECA^-, Ra-LPS) was treated with 0.1N acetic acid as described in the Methods section. The resulting water soluble material was analyzed by liquid chromatography using a Dionex BioLC equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Oligosaccharides were detected by a pulsed amperometric detector (PAD) (Methods). Chromatography conditions are in the described in detail in the Results section. Sodium hydroxide at a concentration of 100mM was added post column to enhance detection.

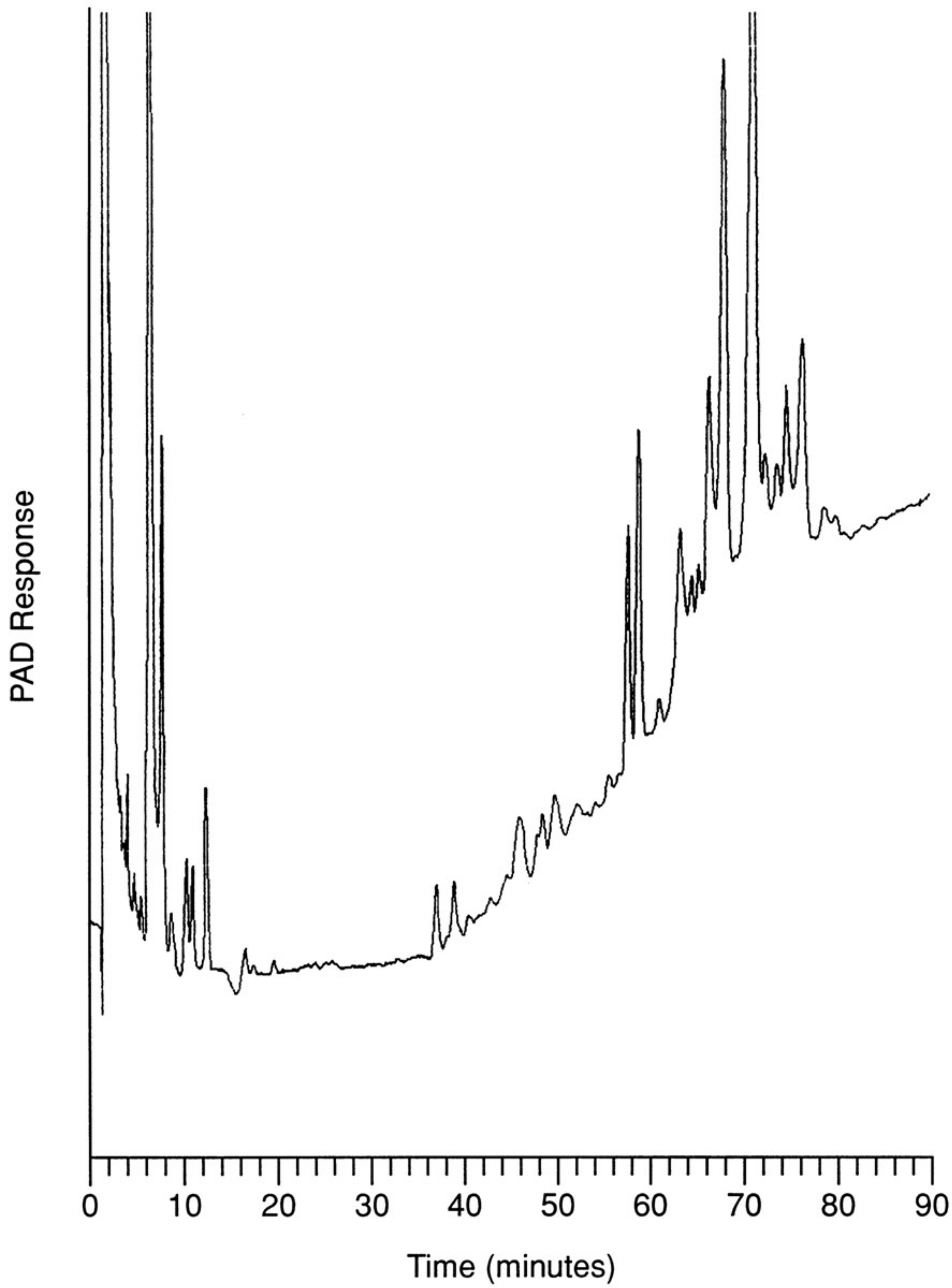
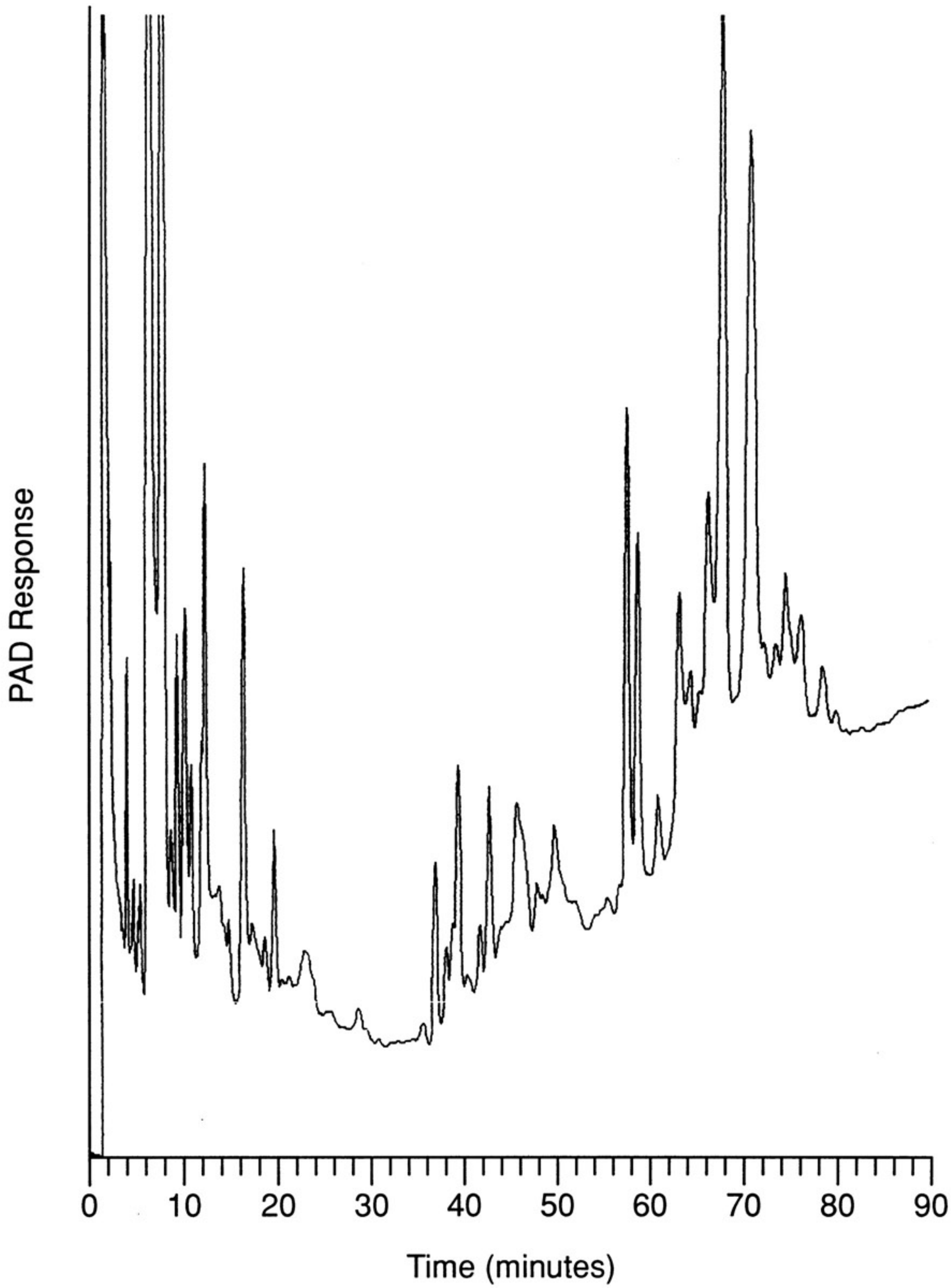
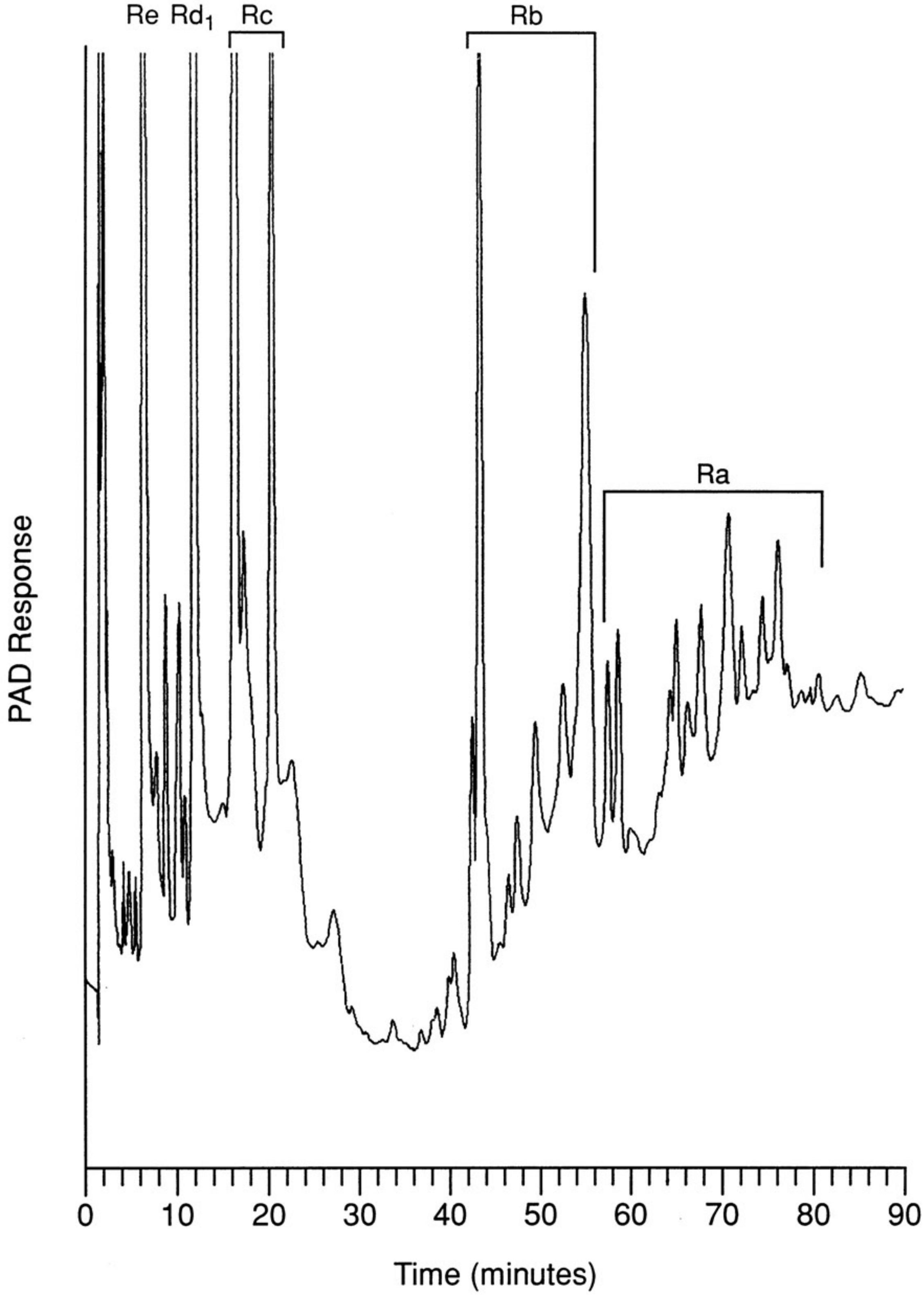

PAD Response
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Figure 34. Analysis of Oligosaccharides Released by Mild Acid from LPS Isolated from HR104. LPS isolated from strain HR104 (ECA-trace, Ra) was treated with 0.1N acetic acid as described in the Methods section. The resulting water soluble material was analyzed by liquid chromatography using a Dionex BioLC equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Oligosaccharides were detected by a pulsed amperometric detector (PAD) (Methods). Chromatography conditions are in the described in detail in the Results section. Sodium hydroxide at a concentration of 100mM was added post column to enhance detection.


PAD Response
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Figures 35. Analysis of Oligosaccharides Released by Mild Acid from LPS-Chemotype Standards. A mixture of purified Ra-, Rb-, Rc-, Rd1- and Re- lipopolysaccharides (List Biological Laboratories, Inc) were treated with 0.1N acetic acid as described in the Methods section. The resulting water soluble material was analyzed by liquid chromatography using a Dionex BioLC equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Oligosaccharides were detected by a pulsed amperometric detector (PAD) (Methods). Chromatography conditions are in the described in detail in the Results section. Sodium hydroxide at a concentration of 100mM was added post column to enhance detection.


Re
Rd1
Rc
Rb
Ra
PAD Response
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Rd_1, and Re chemotypes by mild acid (Figures 36-40). Most prominent are significant peaks at 17 and 21 minutes which match peaks observed in the elution pattern of oligosaccharides released from Rc-chemotype LPS and the group of peaks between 35 and 40 minutes which correspond to the Rb-chemotype pattern. In addition, the peaks at 9, 11, and 17 minutes correspond to peaks observed in the oligosaccharide elution pattern, obtained from Rd_1 chemotype LPS and a very prominent peak at 7 minutes also corresponds to a similar peak seen in the oligosaccharide elution pattern obtained from Re-chemotype LPS. The 7 minute peak is actually observed in all of the patterns, and it is believed to be free KDO which also gives a retention time of 7 minutes in this system (data not shown).

Structure of Lipid II

The structure and nature of lipid II needed to be established to verify that ECA was synthesized via a carrier lipid in the mechanism proposed in figure 4. It was also necessary to determine the precise structure of the carrier lipid, to possibly explain the lipid II role in the ECA-trace phenotype and to help narrow the possible function of the *rfe* gene product in both ECA and O-antigen synthesis.

To determine the structure of lipid II, a procedure for the isolation and purification of this compound first had to be developed. The ECA-trace strain SH5187 (Rick et al., 1988; Mäkelä et al., 1976) was grown in 100 liters of medium A containing 0.2% glucose at 37°C in a 150-liter fermenter (New Brunswick) to an OD_{600nm} of 0.8. The cells were then harvested as described in the Methods section. The final cell pellet was weighed, resuspended and washed one time each with 200ml of 0.9% saline, 200ml of 95% ethanol, and

Figure 36. Analysis of Oligosaccharides Released from Ra-LPS by Mild Acid from LPS-Chemotype Standards. Purified Ra-lipopolysaccharide (List Biological Laboratories, Inc) was treated with 0.1N acetic acid as described in the Methods section. The resulting water soluble material was analyzed by liquid chromatography using a Dionex BioLC equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Oligosaccharides were detected by a pulsed amperometric detector (PAD) (Methods). Chromatography conditions are in the described in detail in the Results section. Sodium hydroxide at a concentration of 100mM was added post column to enhance detection.

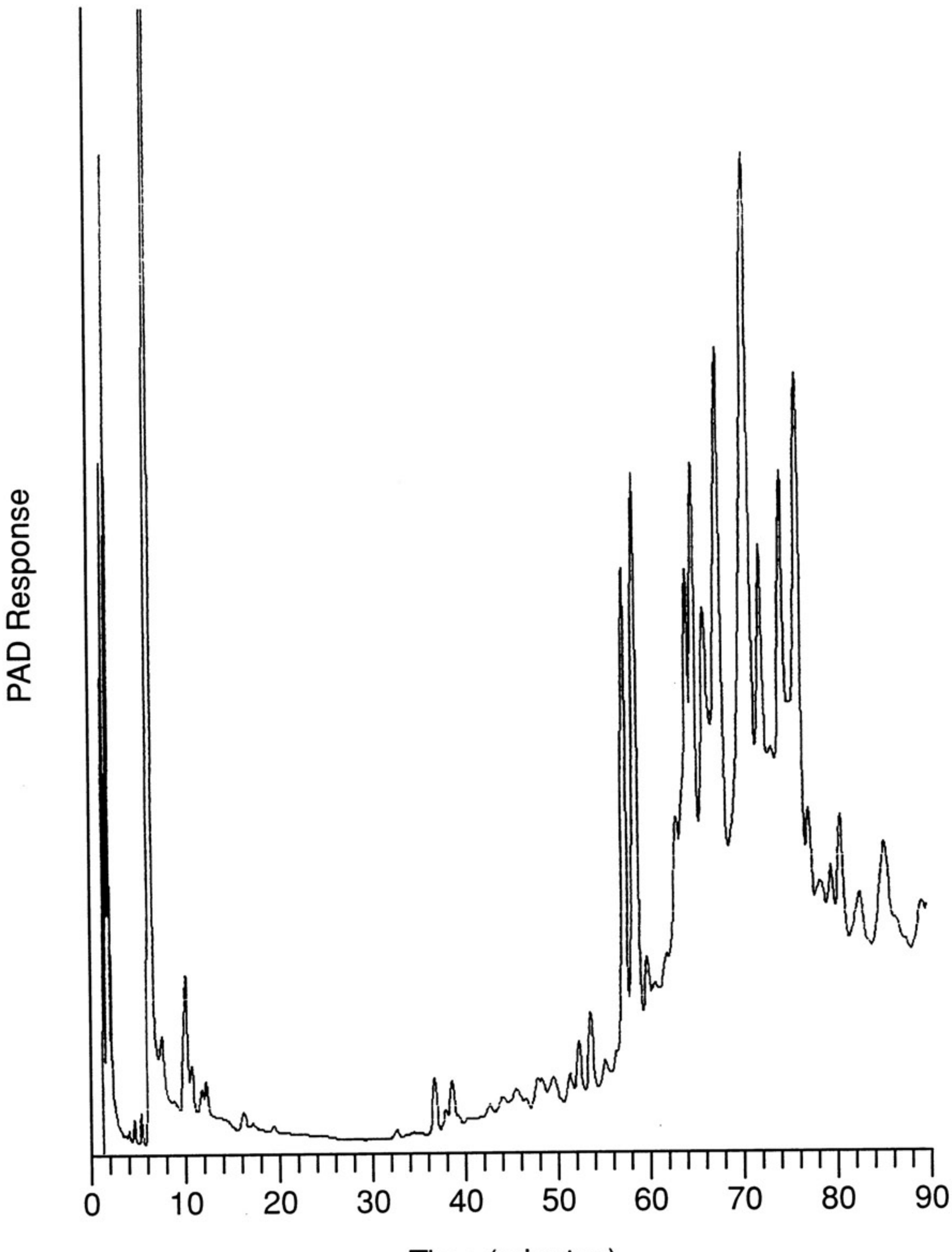
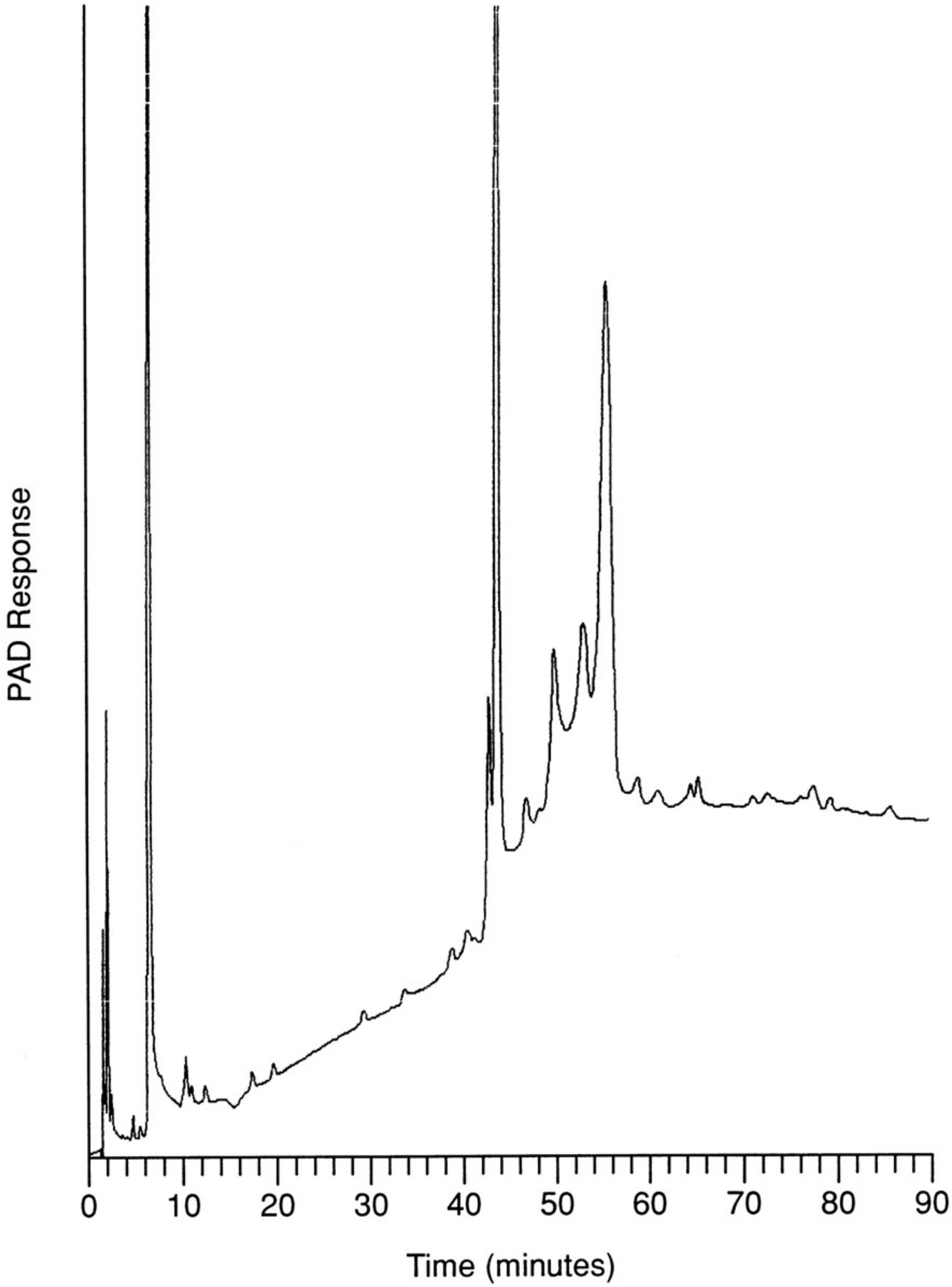

PAD Response
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Figure 37. Analysis of Oligosaccharides Released from Rb-LPS by Mild Acid from LPS-Chemotype Standards. Purified Rb-lipopolysaccharide (List Biological Laboratories, Inc) was treated with 0.1N acetic acid as described in the Methods section. The resulting water soluble material was analyzed by liquid chromatography using a Dionex BioLC equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Oligosaccharides were detected by a pulsed amperometric detector (PAD) (Methods). Chromatography conditions are in the described in detail in the Results section. Sodium hydroxide at a concentration of 100mM was added post column to enhance detection.



PAD Response
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Figure 38. Analysis of Oligosaccharides Released from Rc-LPS by Mild Acid from LPS-Chemotype Standards. Purified Rc-lipopolysaccharide (List Biological Laboratories, Inc) was treated with 0.1N acetic acid as described in the Methods section. The resulting water soluble material was analyzed by liquid chromatography using a Dionex BioLC equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Oligosaccharides were detected by a pulsed amperometric detector (PAD) (Methods). Chromatography conditions are in the described in detail in the Results section. Sodium hydroxide at a concentration of 100mM was added post column to enhance detection.

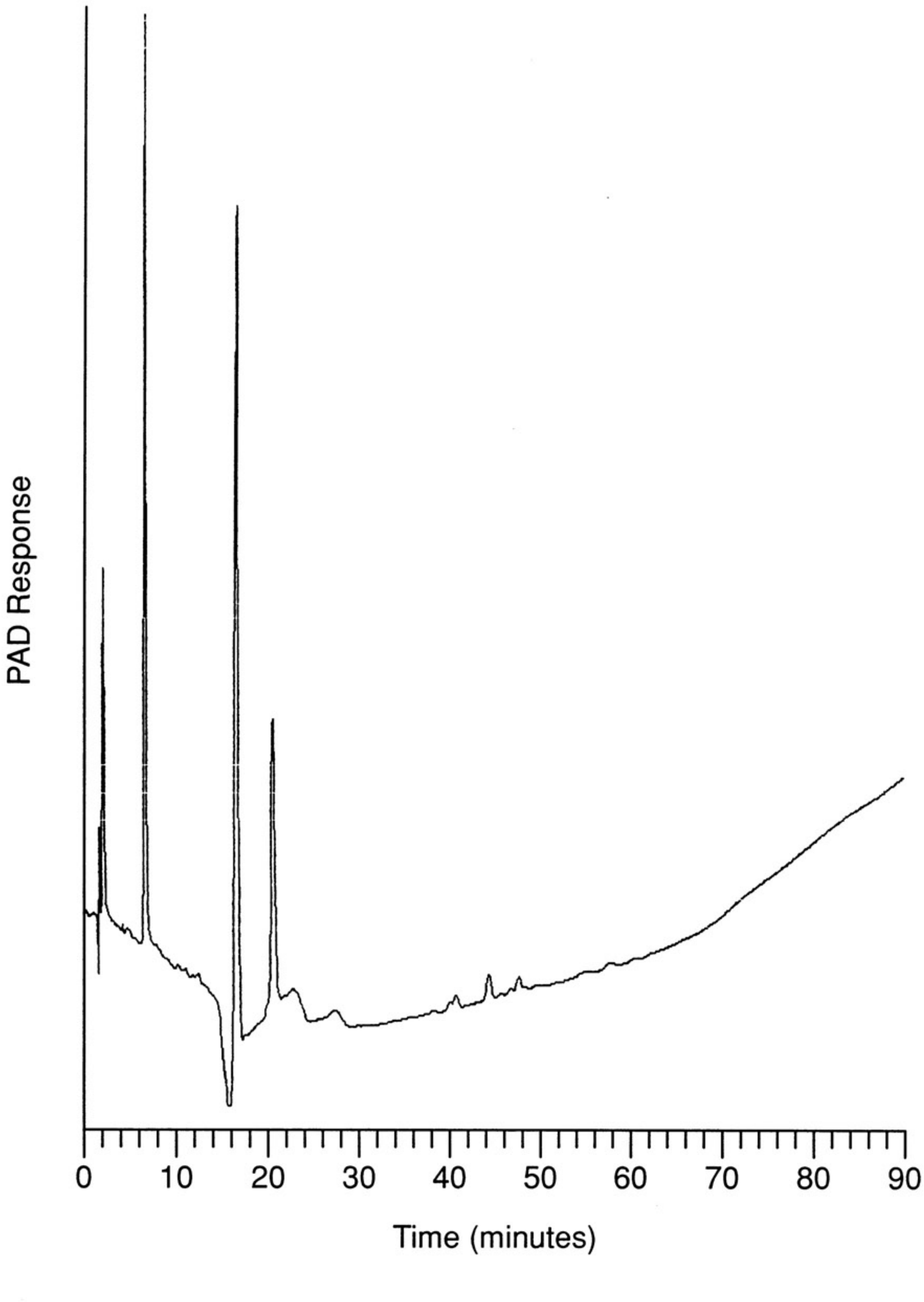

PAD Response
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Figure 39. Analysis of Oligosaccharides Released from Rd1-LPS by Mild Acid from LPS-Chemotype Standards. Purified Rd1-lipopolysaccharide (List Biological Laboratories, Inc) was treated with 0.1N acetic acid as described in the Methods section. The resulting water soluble material was analyzed by liquid chromatography using a Dionex BioLC equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Oligosaccharides were detected by a pulsed amperometric detector (PAD) (Methods). Chromatography conditions are in the described in detail in the Results section. Sodium hydroxide at a concentration of 100mM was added post column to enhance detection.

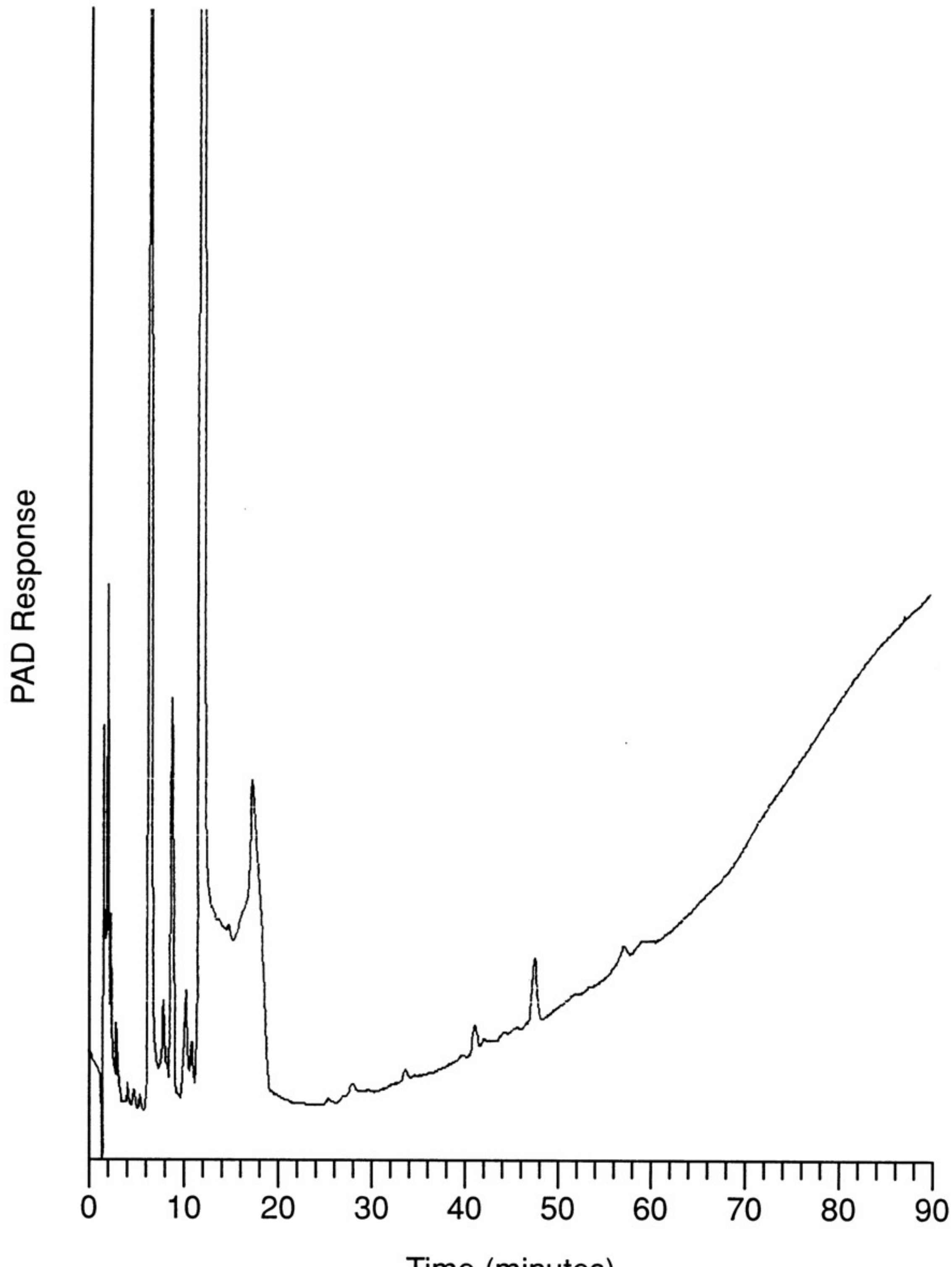
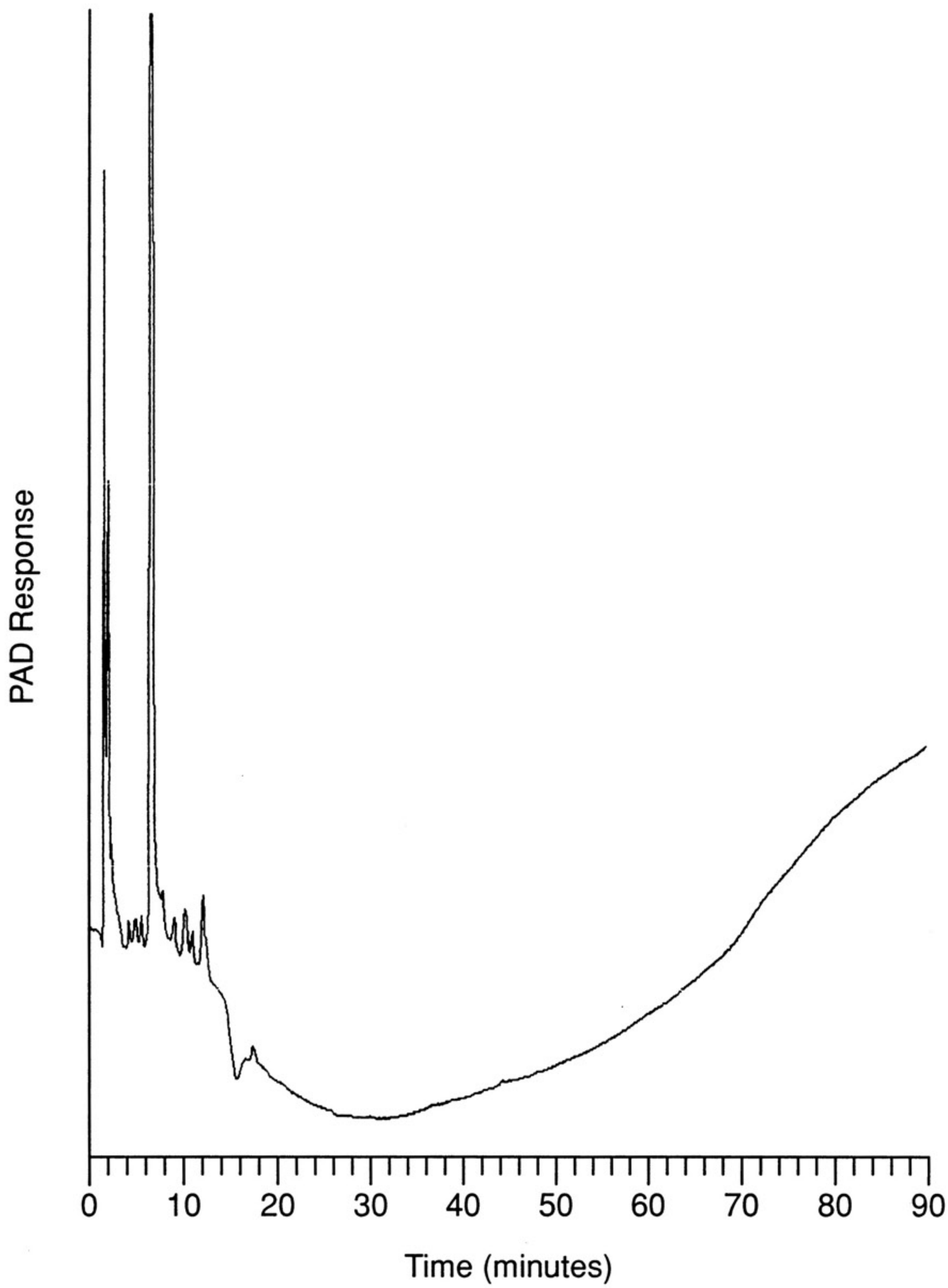

PAD Response
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Figure 40. Analysis of Oligosaccharides Released from Re-LPS by Mild Acid from LPS-Chemotype Standards. Purified Re-lipopolysaccharide (List Biological Laboratories, Inc) was treated with 0.1N acetic acid as described in the Methods section. The resulting water soluble material was analyzed by liquid chromatography using a Dionex BioLC equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Oligosaccharides were detected by a pulsed amperometric detector (PAD) (Methods). Chromatography conditions are in the described in detail in the Results section. Sodium hydroxide at a concentration of 100mM was added post column to enhance detection.



PAD Response
0
10
20
30
40
50
60
70
80
90
Time (minutes)

200ml of acetone. The acetone pellet was dried under vacuum overnight and the dry-weight of the pellet was determined. The material was then stored at -20°C.

Both [^{3}H]GlcNAc-labeled and unlabeled lipid II preparations were purified. Labeled preparations obtained from strain SH5187 were carried through to the acetone powder step, as described in the Methods section for ECA-intermediates, and subsequently combined with the acetone power obtained from unlabeled cells obtained from 100 liters of fermenter grown cells.

The structure of lipid II was believed to be similar to that of the component B intermediate involved in teichuronic acid synthesis (Johnson et al., 1984). Accordingly, modifications of the solvent systems and isolation procedures used for the purification of compound B were used for the purification of lipid II. The pellet was extracted once with butanol/pyridinium acetate, (BPA) (2:1, v/v) using 4ml BPA/gm of dry-weight pellet. The residue was extracted again with 50% of the first BPA volume. The BPA extracts were combined and the exact volume was determined. An equal volume of methanol was then added, and the extract was centrifuged at 10,000xg to remove precipitated material. The supernatant was then loaded onto a DEAE-acetate column (3x10cm) that had been pre-equilibrated with methanol/ butanol/ pyridinium acetate, (methanol/BPA) (3:2:1, v/v/v). After loading, the column, was washed with 200ml of methanol/BPA. The column was then washed with 200ml of methanol/pyridinium acetate (1:4, v/v) followed by 200ml of methanol and 200ml $CHCl_3$/methanol/water (2:3:1, v/v/v). Lipid II was then eluted with 200ml 0.1<u>M</u> ammonium acetate in $CHCl_3$/methanol/water ((2:3:1, v/v/v). Approximately 6ml fractions were collected. The radioactivity eluted in a

broad, perhaps double peak (Figure 41) and the fractions from this peak were pooled and evaporated to dryness. The residue was then resuspended in 1.0ml $CHCl_3$/methanol/water (10:10:3, v/v/v) and loaded onto a Fractogel column (1.5x31cm) equilibrated with methanol. The column was eluted with methanol and 2ml fractions were collected (Figure 42). The radioactivity eluted early as a single sharp peak and fractions containing this peak were pooled and evaporated to dryness under reduced pressure.

The location of [^{3}H]GlcNAc-labeled material in the labeled preparations was determined by counting 100ul aliquots of each fraction. For unlabeled preparations, peaks were determined by spotting 100ul of each fraction onto channeled LK5 TLC plates that were subsequently developed in solvent B. The dried developed plates were then sprayed with cupric acetate reagent and ashed. Material for further analysis was obtained from fractions showing material at Rf=0.28.

The purity of the final material was determined by two-dimensional thin-layer chromatography by spotting 10ul of the sample on the corner of a LK5 TLC plate (10x10cm) and developing the plate using solvent B in the first dimension and solvent A in the second dimension. After the plate was developed and dried, it was sprayed with cupric acetate reagent and ashed to determine the location of organic material.

Purified material was dried under a nitrogen stream and then stored under nitrogen at -80°C in a sealed tube.

Purification yields are presented in Table 10. The amount of lipid II in the final preparations was determined by assaying the phosphate content of the purified material assuming a "single" compound with a molecular weight of 1346 having two phosphates per molecule. Additional phosphate-

Figure 41. Purification of Lipid II by DEAE-Cellulose Column Chromatography of the BPA-extracts of ECA-trace Strain SH5187. butanol/ pyridinium acetate, BPA (3:2, v/v) extracts of SH5187 (previously combined with [^{3}H]GlcNAc labeled cells) were combined, diluted with methanol and centrifuged (Results). The supernatant was then loaded onto a DEAE-acetate column (3x10cm) that had been equilibrated with methanol/ butanol/ pyridinium acetate, methanol/BPA (3:2:1, v/v/v). The column, was then washed with 200ml of methanol/BPA. The column was next washed with 200ml of methanol/PA (1:4, v/v) followed by 200ml of methanol and 200ml $CHCl_3$/methanol/water (2:3:1, v/v/v). Lipid II was then eluted with 200ml 0.1M ammonium acetate in $CHCl_3$/methanol/water (2:3:1, v/v/v). Fractions containing approximately 6ml were collected. Radioactivity was determined by counting100ul from each fraction.

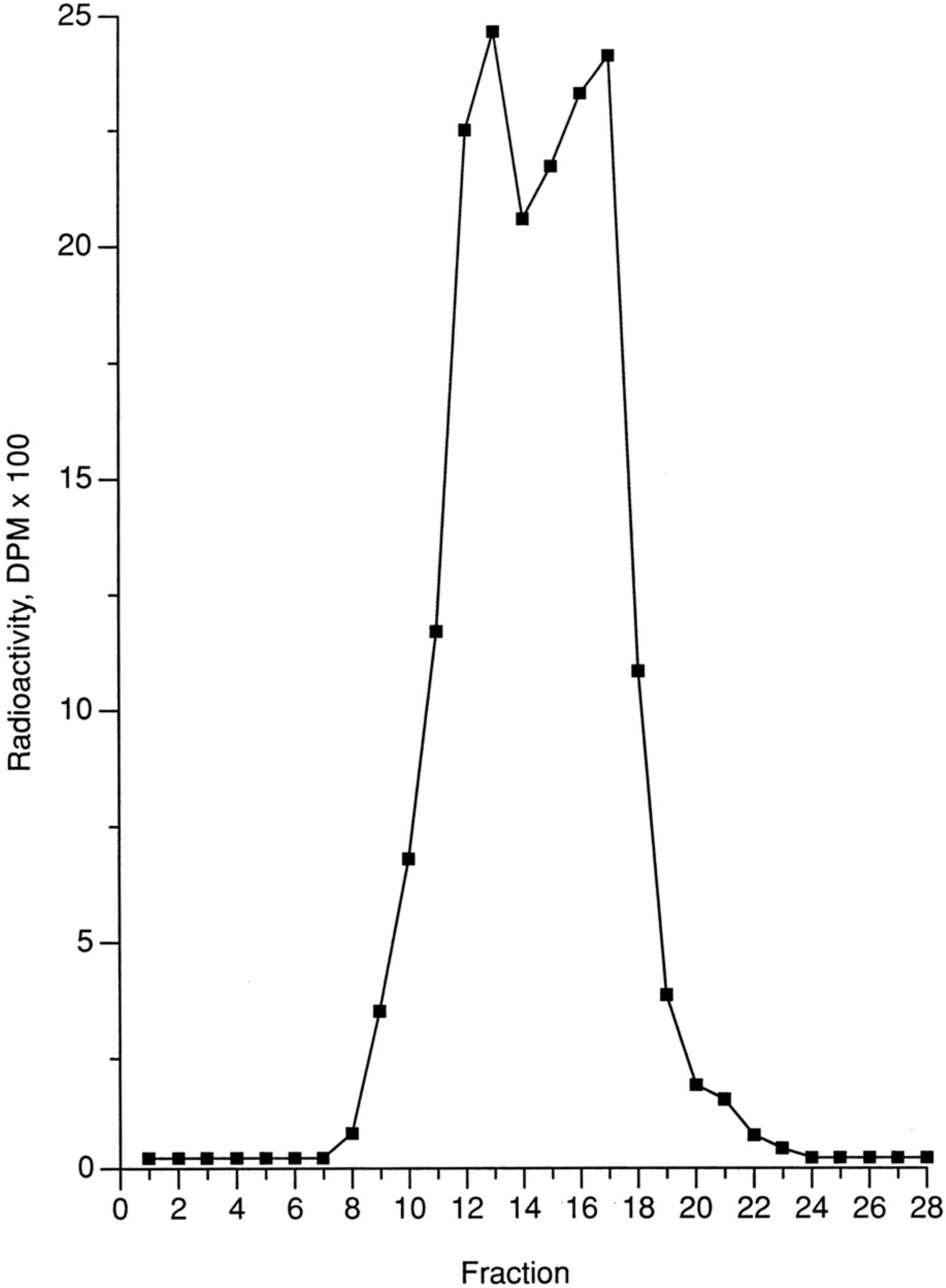

25
20
15
10
5
0
Radioactivity, DPM x 100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Fraction

Figure 42. Gel Filtration Column Chromatography of [^{3}H]GlcNAc-Labeled Lipid. DEAE column fractions containing the [^{3}H] labeled material (see Figure 41) were pooled and dried under reduced pressure. The residue was resuspended in 1.0ml $CHCl_3$/methanol/water (10:10:3, v/v/v) and loaded onto a Fractogel column (1.5x31cm) equilibrated with methanol. The column was eluted with methanol and 2ml fractions were collected. Radioactivity was determined by counting 50ul of each fraction.

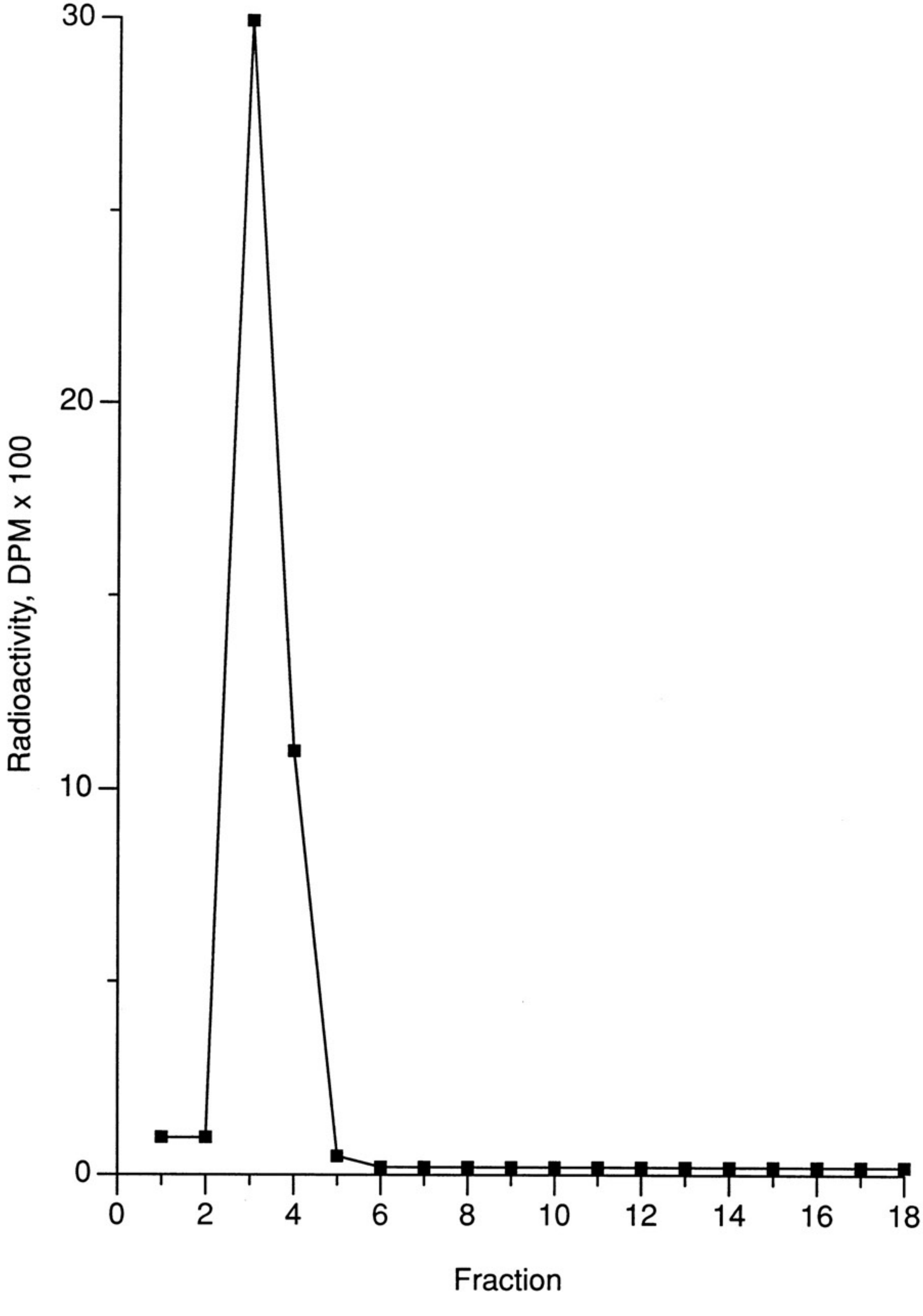

30
20
10
0
Radioactivity, DPM x 100
0
2
4
6
8
10
12
14
16
18
Fraction

Table 10. Lipid II Isolation Yields *

Preparation	Cell Wet Wt.	Cell Dry Wt.	ug Lipid II	Yield(dry wt.)
^{3}H-Lipid II	161gm	33.4gm	39ug	1.2ug/gm
Lipid II	57gm	12.4gm	170ug	13.7ug/gm

* Lipid II was isolated as specified in the results section. The amount of lipid II was determined from the organic phosphate content (Methods) of the final material assuming 2 moles of phosphate per mole of lipid II.

containing compounds present would obviously produce a much higher estimate of purified product (unlabled material, Table 10).

The purity of a [^{3}H]-lipid II preparation is shown in Figure 43. The two-dimensional thin-layer chromatogram shows only a single component. However, two-dimensional thin-layer chromatography of purified unlabeled material also showed a second component which migrated faster but never resolved completly from the first component, and whose relationship to lipid II was unclear (not shown).

The chromatographic mobility of the purified preparation was also compared with that of a lipid II standard (Rick et al., 1988) as determined by SG-81 paper chromatography (Figure 44) and LK5 -thin layer chromatography. The [^{3}H]lipid II preparation was also analyzed by Bio-Gel P2 chromatography of the oligosaccharide released by mild acid (see Methods). The water-soluble tritiated material eluted from the column in approximately in the same fraction that was observed for the disaccharide, chitobiose (Figure 45). These data serve as additional evidence that this material was indeed lipid II (Figure 45).

The purified material was submitted to Dr. Masahiro Kitaoka (Sankyo Company, Tokyo, Japan) for fast atom bombardment-tandem mass spectrometry (FAB-MS/MS) analysis. The combination of tandem mass spectrometry (MS/MS) with FAB-MS has provided detailed and total analyses of the structures of similar isoprenoid compounds (Kitaoka et al., 1990). An advantage of the FAB/MS/MS analytical technique is that the spectrum of a compound can still be obtained from a mixture by secondary analysis of individual molecular ion fragments obtained from the initial fragmentation (Kitaoka et al., 1990).

Figure 43. Two-Dimensional Thin Layer Chromatography of Isolated Lipid II. The purity of isolated lipid II was determined by two-dimensional thin-layer chromatography. The sample was resuspended in 1.0ml of chloroform/methanol/water (10:10:3, v/v/v) and 10ul was spotted on the corner of a LK5 TLC plate (10x10cm). The plate was developed using solvent B in the first dimension and solvent A in the second dimension. After development, the plate was dried, sprayed with cupric acetate reagent and heated at 210^{o}C for 5 minutes.

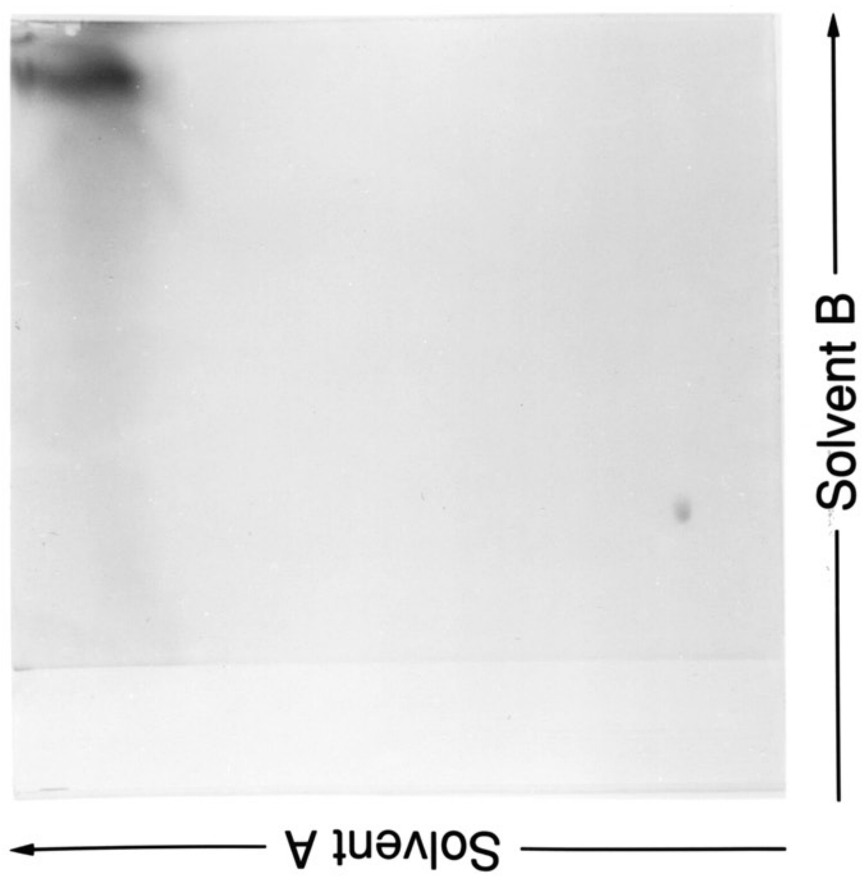
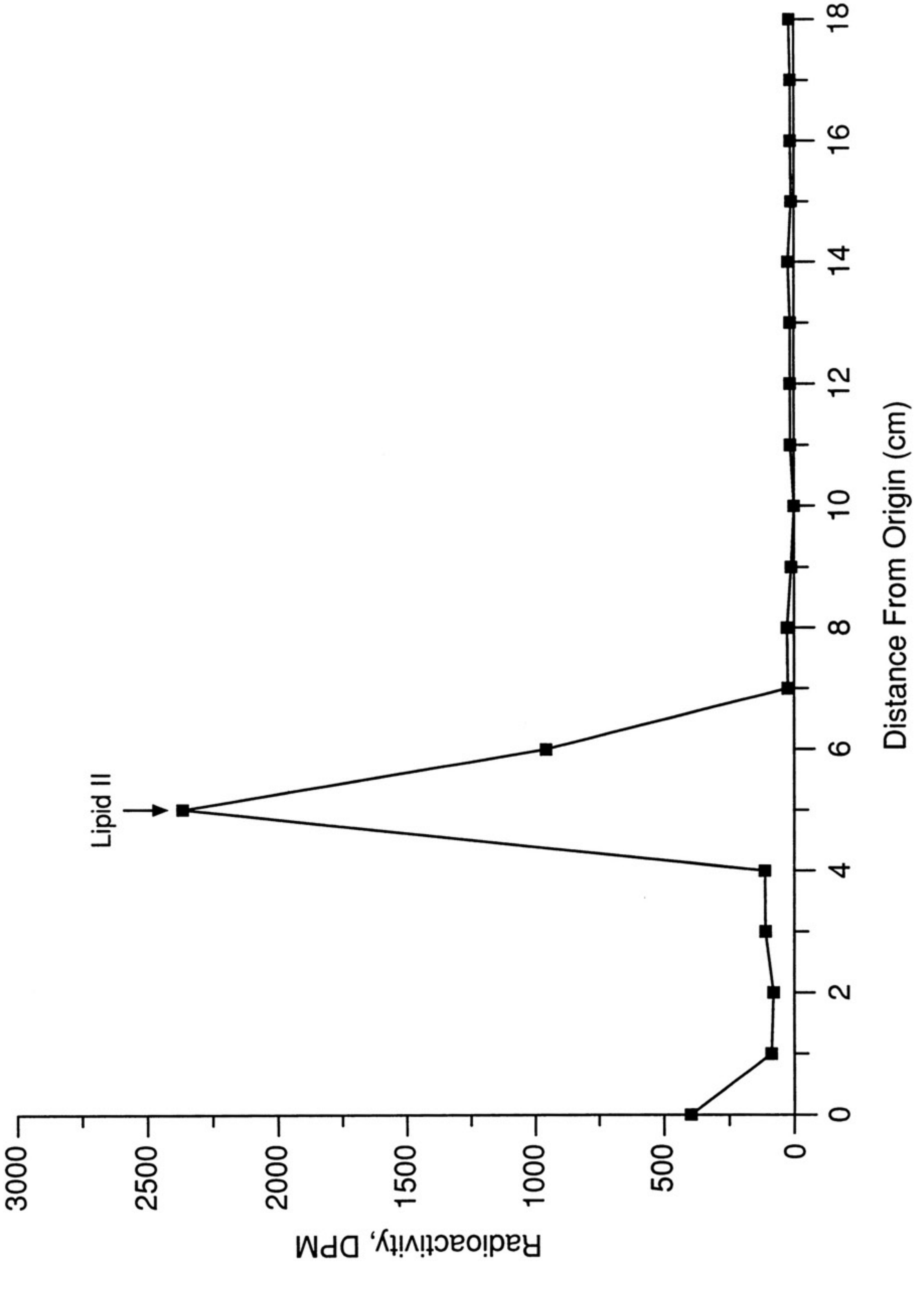
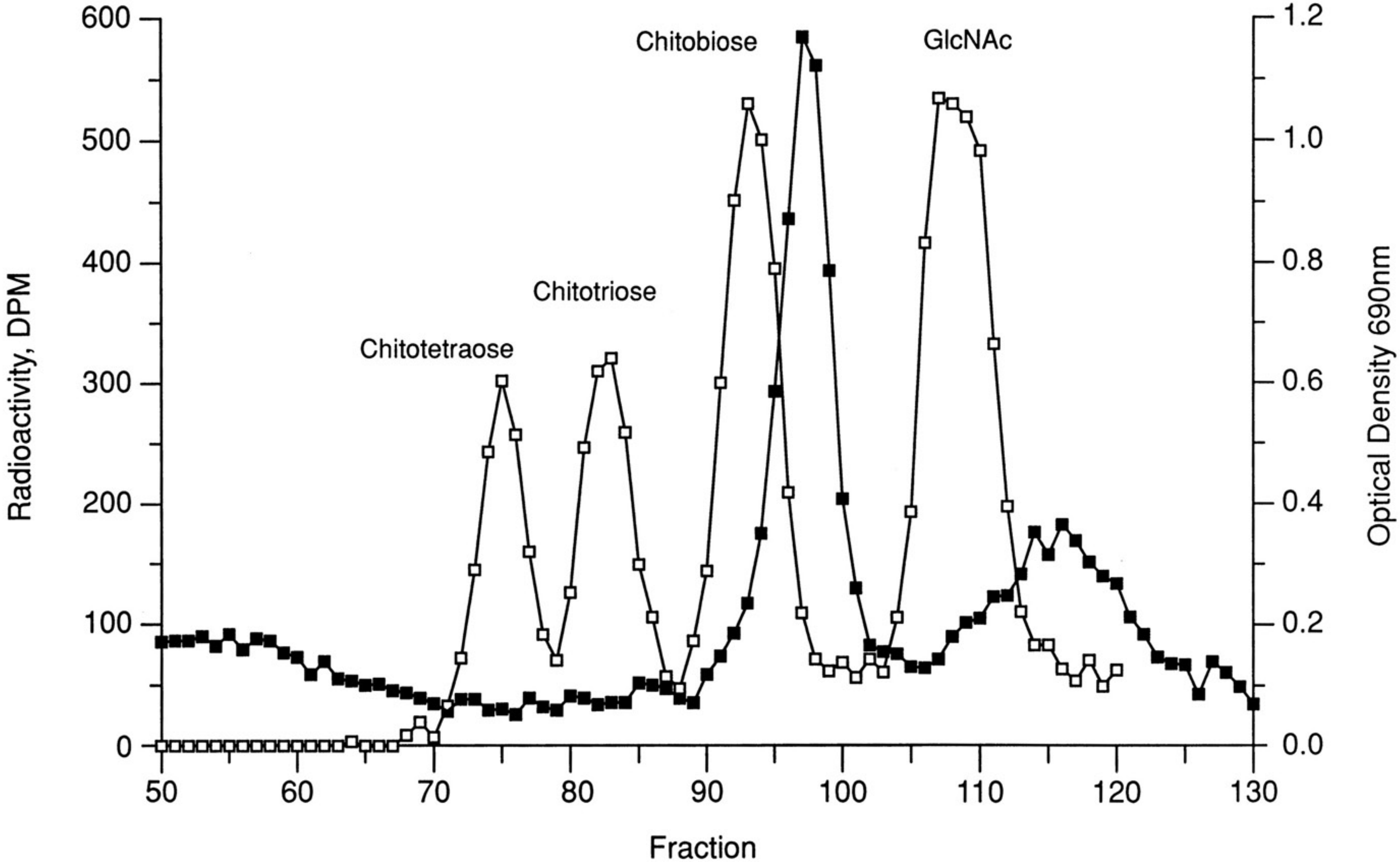

Solvent B
Solvent A

Figure 44. SG81 Paper Chromatography of [^{3}H]GlcNAc-labeled Lipid II. The purity and identity of purified [^{3}H]GlcNAc-labeled lipid II was determined SG81 paper chromatography. The sample was resuspended in 1.0ml of chloroform/methanol/water (10:10:3, v/v/v) and 10ul was spotted on the paper. The chromatogram was developed in solvent B and prepared for counting as described in the Methods section. Lipid II standard was partially purified by DEAE-chromatography as detailed in the Methods section and applied to a separate lane on the SG81 chromatogram.



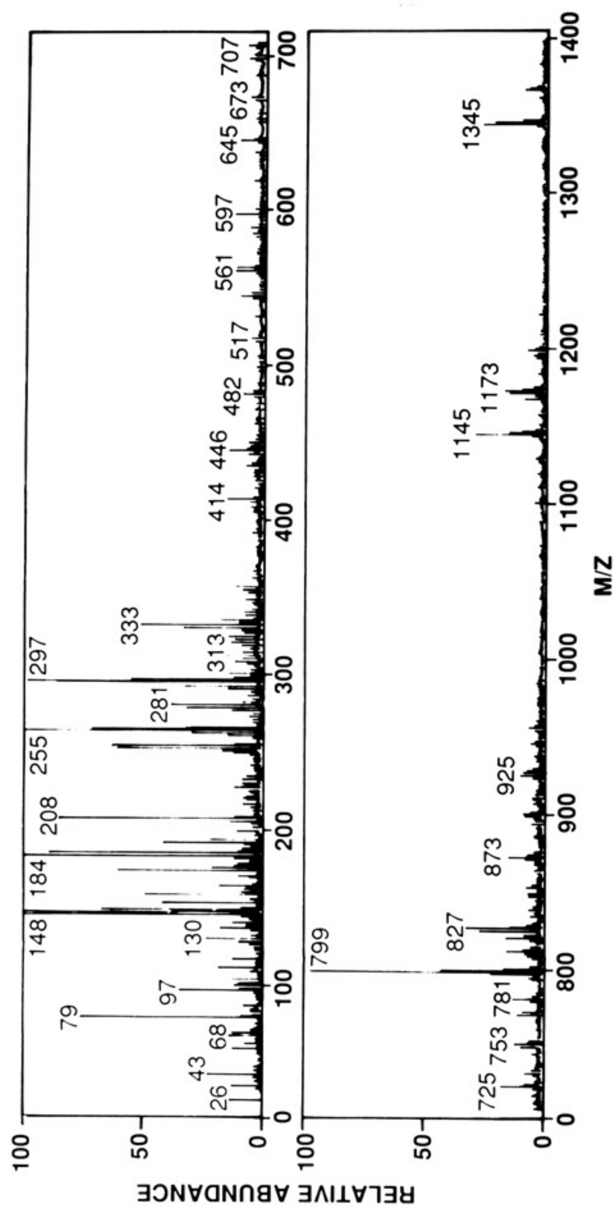
Lipid II
Radioactivity, DPM
0
500
1000
1500
2000
2500
3000
Distance From Origin (cm)
0
2
4
6
8
10
12
14
16
18

Figure 45. Bio-Gel P2 Chromatography of the Radioactive Component Released from the Purified Lipid II by Mild-Acid Hydrolysis. Purified [^{3}H]GlcNAc-labeled lipid II was resuspended in 1.0ml of chloroform/methanol/water (10:10:3, v/v/v) and 50ul was applied across the loading area of an SG81 paper. The chromatogram was subsequently developed in solvent B. A narrow lane of the chromatogram was sectioned and counted to locate the radioactive lipid II. The remaining area of the paper containing the radioactive lipid II was eluted with solvent C and collected. The solvent was removed under a stream of nitrogen and the residue was acid hydrolyzed and subjected to Bio-Gel P2 chromatography as described in Methods. Radioactivity was determined by counting 0.5ml of each fraction. GlcNAc (N-acetylglucosamine), chitobiose ($GlcNAc_2$) chitotriose ($GlcNAc_3$) and chitotetraose ($GlcNAc_4$)standards (1 umole each) were added to the the sample prior to loading on the column. The elution pattern of these standards was determined by assaying 200ul of each fraction using the Ferricyanide-Reducing sugar assay (Methods). ■, radioactivity; □, reducing sugar.


Chitotetraose
Chitotriose
Chitobiose
GlcNAc
Radioactivity, DPM
Optical Density 690nm
Fraction
600
500
400
300
200
100
0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
50
60
70
80
90
100
110
120
130

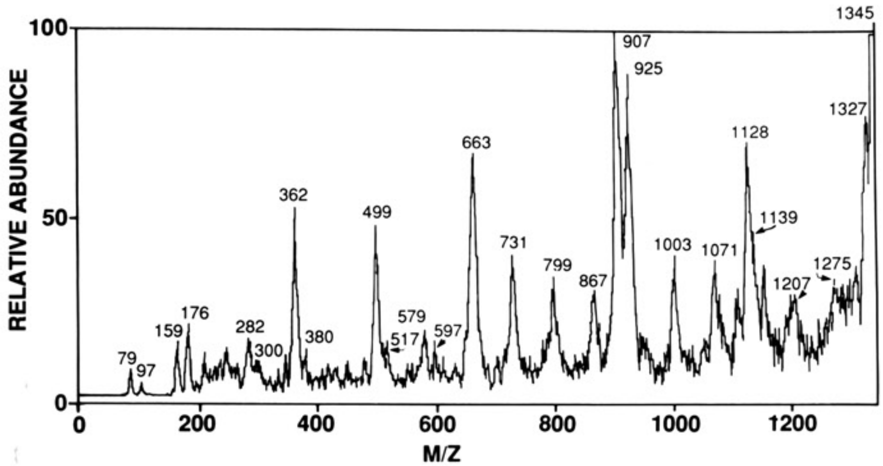
The results from analysis of the initial FAB/MS spectrum of the purified unlabeled material identified several compounds yielding molecular ions of 1345, 1173, 1145, and 799 (Figure 46). Additional molecular ion species were also observed which were smaller fragments of these four parent ions. The FAB/MS spectrum analysis of the purified [^{3}H]-labeled material identified only two compounds; the 1345 and 1145 molecular ion species as well as smaller fragments derived from these (data not shown). For technical reasons only the fragments from the unlabeled sample were further analyzed by secondary MS. The isolated secondary spectrum of the 1345 molecular ion species is shown in Figure 47 along with the deduced structure based on the fragment ions. Compound 1345 is a C55-undecaprenol linked via pyrophosphate to the reducing terminus of GlcNAc on a ManNAcA-GlcNAc dissaccharide. This was the expected structure for lipid II and the fragment ions present in the spectrum are consistent with this structure (Figure 47). The 1145 species also contains the dissaccharide pyrophosphate, but the deduced structure based on the fragment ions is consistent with linkage of a disaccharide pyrophosphate to a diglyceride (Figure 48). The fatty acid composition seems to be 16:0 in the C-1 position and 16:1 in the C-2 position. A complete secondary spectrum for the 1173 species has not been completed, as there was a reduced amount of this compound. However, the preliminary data suggests that this compound is the same as the 1145 species with the exception of an 18:1 fatty acyl substituent at the C-2 position. Two additional saturated carbons would put the initial molecular ion to 1173. The 799 species was relatively abundant in the unlabeled sample and the secondary MS spectrum revealed this to be phosphatidylglycerol phosphate (PGP) (data not shown). This compound may account for the second component observed in the unlabled lipid II two-

Figure 46. Negative Ion FAB-MS Spectrum of the Unlabeled Purified Lipid II Preparation. The sample was dissolved in an aliquot of glycerol and 0.5ug phosphate equivalent was applied to the FAB probe. The spectrum was obtained with a JEOL JMS-HX 100 triple analyzer tandem mass spectrometer (Kitaoka et al., 1990). Xenon provided the primary atom beam. Note ion peaks 1345, 1145 and 799 which were subjected to further analysis by collisionally activated dissociation (CAD) spectroscopy.



RELATIVE ABUNDANCE
100
50
0
M/Z
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
26
43
68
79
97
130
148
184
208
255
281
297
313
333
414
446
482
517
561
597
645
673
707
725
753
781
799
827
873
925
1145
1173
1345

Figure 47. Secondary MS Spectrum (CAD) of the 1345 FAB/MS Peak Obtained from Lipid II. A secondary spectrum of the molecular ion having a M/Z=1345 was obtained with a JEOL JMS-HX 100 triple analyzer tandem mass spectrometer. The molecular ion species produced from the original collisions was activated by collisions with argon gas to obtain the collisionally activated dissociation (CAD) spectrum (Kitaoka et al., 1990). The lower portion of the figure shows the origin of individual ion fragments.


RELATIVE ABUNDANCE
M/Z
1345
907
925
1327
1128
663
362
499
1139
731
1003
1071
799
1275
867
1207
176
579
282
159
597
300
380
517
79
97

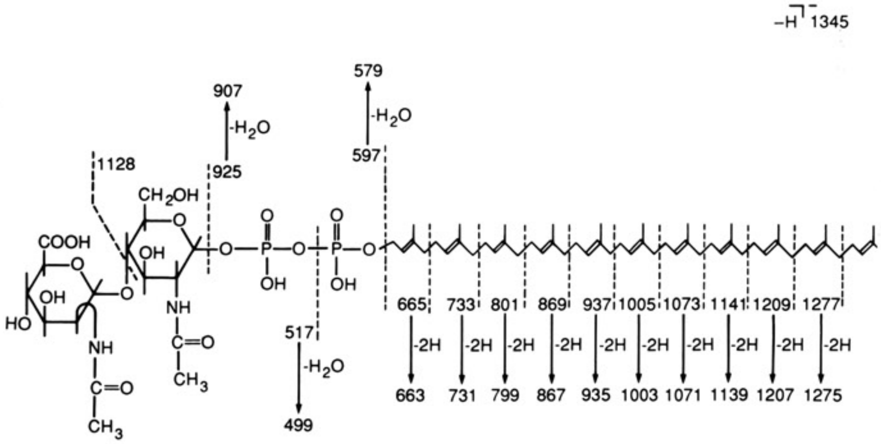
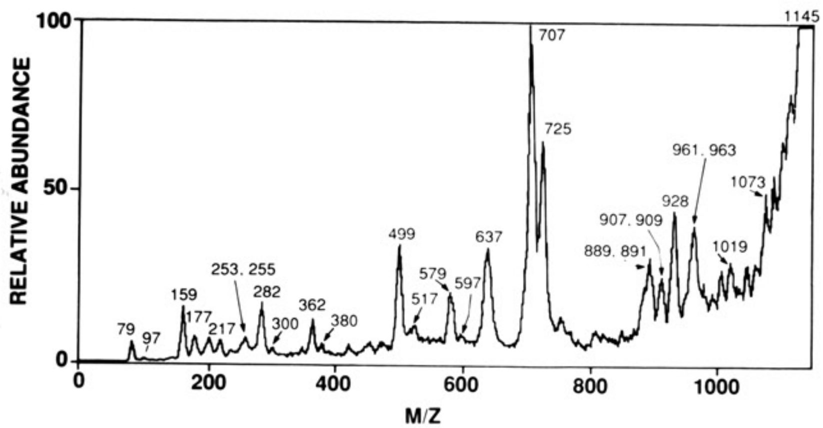
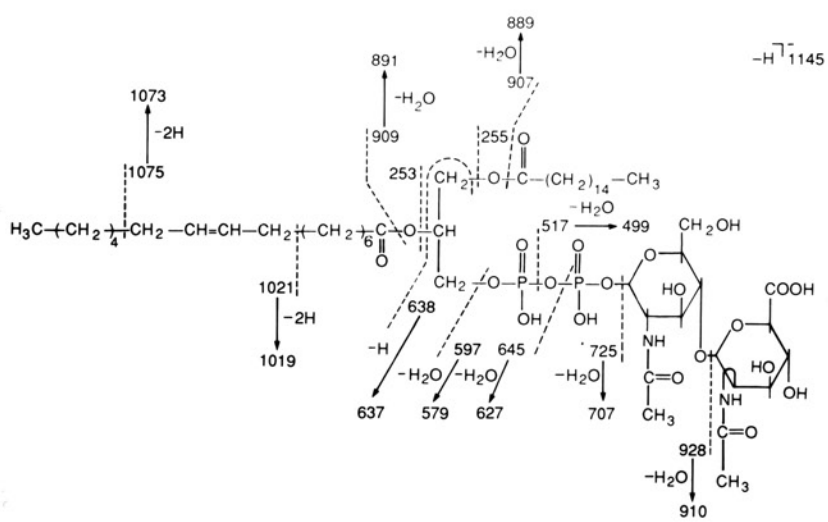

-H 1345
579
-H2O
597
907
-H2O
925
1128
CH2OH
COOH
OH
OH
HO
NH
C=O
CH3
OH
OH
517
-H2O
499
665 733 801 869 937 1005 1073 1141 1209 1277
-2H
663 731 799 867 935 1003 1071 1139 1207 1275

Figure 48. Secondary MS Spectrum (CAD) of the 1145 FAB/MS Peak Obtained from Lipid II. A secondary spectrum of the molecular ion having a M/Z=1145 was obtained with a JEOL JMS-HX 100 triple analyzer tandem mass spectrometer. The molecular ion species produced from the original collisions was activated by collisions with argon gas to obtain the collisionally activated dissociation (CAD) spectra (Kitaoka et al., 1990). The lower portion of the figure shows the origin of individual ion fragments.


RELATIVE ABUNDANCE
M/Z
707
725
1145
961, 963
928
1073
907, 909
889, 891
1019
499
637
579
597
517
253, 255
282
159
177
217
362
380
300
79
97


−H]⁻ 1145
1073
−2H
1075
1021
1019
891
−H2O
909
889
907
255
253
517 → 499
638
−H
637
597
−H2O
579
645
−H2O
627
725
−H2O
707
928
−H2O
910

dimensional TLC and certainly accounts for the apparent high yield determination for this purification (Table 10).

Role of the *rfe* Gene in the Synthesis of O8 Side Chains in *E. coli*

The results of earlier investigations strongly support the conclusion that the *rfe* gene product is a tunicamycin-sensitive GlcNAc-1-phosphate transferase (Meier-Dieter et al., 1990). Accordingly, lipid I synthesis is abolished in tunicamycin-treated cells (Barr and Rick, 1987). The *rfe* gene is also involved in the biosynthesis of certain O-side chains of *E. coli* and *Salmonella* (Schmidt et al., 1976; Mäkelä et al., 1970). However, the role of the *rfe* gene O side-chain synthesis remains to be established. Thus, experiments were conducted in order to determine the role of the GlcNAc-1-phosphate transferase in the synthesis of the *rfe*-dependent O side-chain of *E. coli* O8.

The synthesis of ECA in *E. coli* and *S. typhimurium* strains that synthesize LPS molecules possessing sugars distal to the first glucosyl residue of the core is resistant to the antibiotic, tunicamycin. In contrast, the synthesis of ECA in mutants lacking all sugars distal to the first glucosyl residue of LPS, as well as in "deep-rough" mutants, is inhibited by tunicamycin. It is believed that the permeability of the outer membrane to tunicamycin is increased in the latter mutants by a mechanism similar to that proposed by Nikaido and co-workers (Nikaido and Vaara, 1985; Rick et al., 1988). In the absence of exogenously supplied glucose, the synthesis of lipid I in *pgi* (phophoglucose isomerase) mutants is also inhibited by tunicamycin (unpublished observation). This follows from the fact that such mutants are unable to synthesize glucose; and as a result, they synthesize a

"deep-rough" LPS which renders them susceptible to tunicamycin. Thus it was reasoned that if lipid I was involved in the synthesis of O8 side-chains, the synthesis of these O side-chains would also be sensitive to tunicamycin in *pgi* mutants grown in the absence of glucose. Accordingly, an *E. coli* O8 strain possessing lesions in *pgi* and *manA* (phosphomannose isomerase) was constructed (Figure 49) in order to examine this possibility. A mutation in *pgi* would not only facilitate inhibition of lipid I synthesis by tunicamycin as stated above, but the subsequent addition of glucose would initiate the synthesis of a complete LPS core to act as an acceptor of O8 side-chains. The O8 side-chain is a polymannan, and a mutation in *manA* would prohibit de novo mannose synthesis (Figure 3) and thereby preclude O8 side-chain synthesis. However, the addition of mannose to the mutant would permit O8 side-chain synthesis. A *manA* lesion would also allow specific labeling of O8 side chains by exogenously supplied radiolabeled mannose.

The final construct, strain HR193 was screened for O8 by cross streaking against phage Ω8 (O8 specific phage). The strain was also found to be unable to utilize both glucose and mannose as the sole source of carbon. Both phosphoglucose isomerase (PGI) and phosphomannose isomerase (PMI) activities were absent or reduced in this strain when compared to controls (Table 11).

Western blot analysis of extracts obtained from HR193 showed that this strain was ECA- when grown in medium C with 2% glycerol and in the absence of exogenously supplied glucose (Figure 50). The addition of glucose restored ECA synthesis, but this was inhibited if tunicamycin was added prior to the addition of glucose. An identical result was obtained when glucose-6-phosphate was substituted for glucose. This substitution became important in subsequent experiments as the presence of glucose would

Figure 49. Construction of a *pgi, pmi* derivative of *E. coli* O8 Strain. Transductions were performed as described in the Methods section. Tetracycline resistant transductants were selected by plating transduction mixtures on medium B containing tetracycline at a final concentration of 25ug/ml. The transductants were screened for mannose and glucose utilization by plating on BTB and TZ plates supplimented with either glucose or mannose at a final concentration of 1% (TZ) or 0.5% (BTB). Fusaric acid selection for the loss of tetracycline resistance was as described in the Methods section. The selection of his^+ prototrophs was performed on medium C plates lacking histidine and containing 2% glycerol as the carbon source. Tn10 linkage to *manA* in strain G146 is 25%.

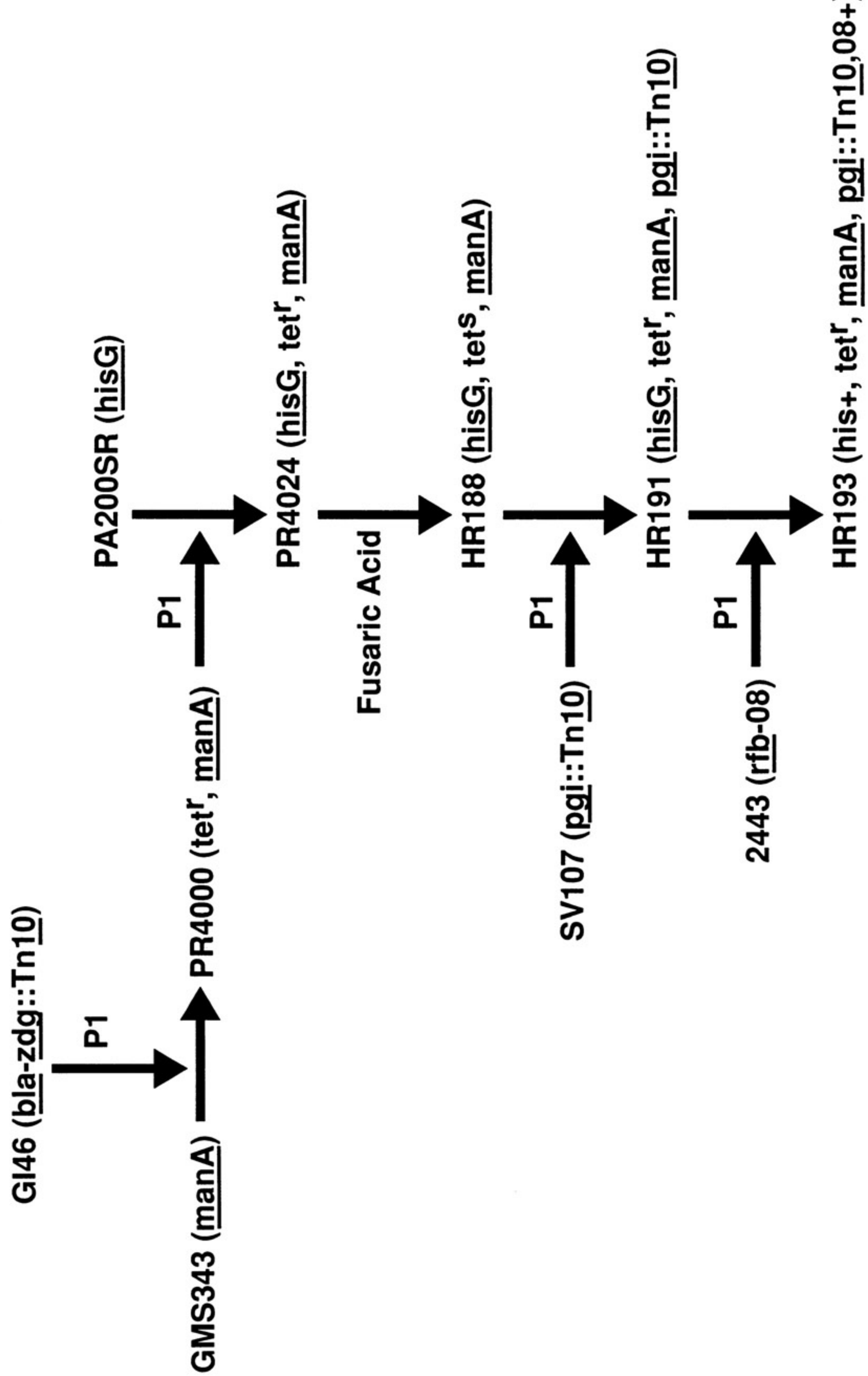
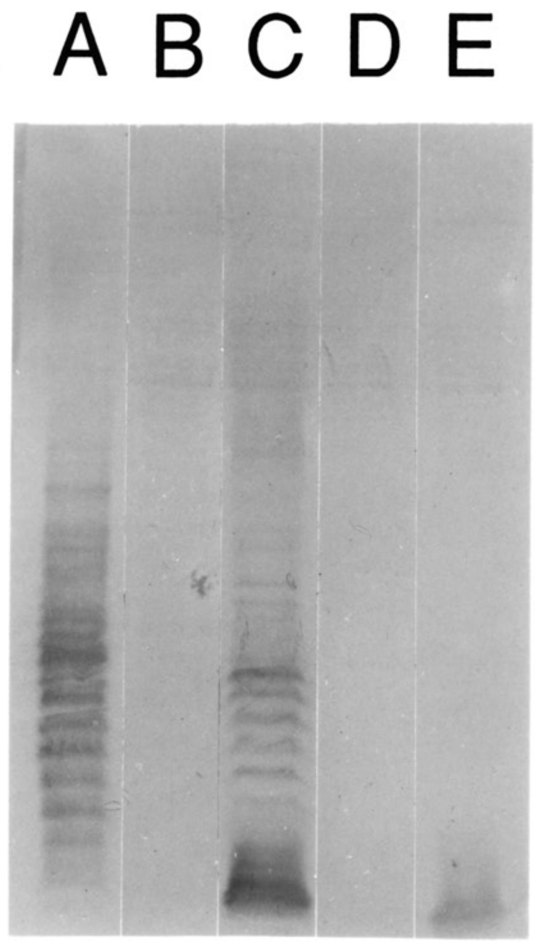

GI46 (bla-zdg::Tn10)
P1
GMS343 (manA)
PR4000 (tet^{r}, manA)
P1
PA200SR (hisG)
PR4024 (hisG, tet^{r}, manA)
Fusaric Acid
HR188 (hisG, tet^{s}, manA)
P1
SV107 (pgi::Tn10)
HR191 (hisG, tet^{r}, manA, pgi::Tn10)
P1
2443 (rfb-08)
HR193 (his+, tet^{r}, manA, pgi::Tn10,08+)

Table 11: Specific Activities of Phosphoglucose Isomerase and Phosphomannose Isomerase in Extracts of Strain HR193

Strain	Relevant Genotype	Specific Activity * PMI	PGI	G6PDH
PA200	Wild type	190	64	57
HR193	(O8,*pmi, pgi*)	40	2	84

* Activities are expressed as nmole minute^{-1} mg^{-1}. Enzymes were assayed as described in the Methods. Protein was assayed by the BCA method. Abbreviations: PMI, phosphomannose isomerase; PGI, phosphoglucose isomerase; G6PDH, glucose-6-phosphate dehydrogenase.

Figure 50. Effect of Exogenously Supplied Glucose on the Ability of Strain HR193 to Synthesize ECA. Strain HR193 (*manA*, *pgi*::Tn10) was grown overnight at 37°C in medium C with 2% glycerol as the sole carbon source. Five-milliliters of the overnight culture was transferred to two flasks containing 50ml of fresh medium C supplemented with 2% glycerol. To one flask was added 0.5ml of a 1mg/ml tunicamycin solution (in 25mM NaOH), and the cultures were incubated at 37°C for 4-5 hours until an OD_{600nm} of 0.2 was reached. An additional 500ul of tunicamycin solution was then added to the tunicamycin containing flask and the cultures were incubated for an additional 15 minutes. The cultures were next subdivided to give three 10ml subcultures, and two of each set of subcultures were supplemented with either 50ul of 40% glucose or 40% glucose-6-phosphate. The cultures were then incubated for one hour at 37°C. The cells were then harvested and analyzed for the presence of ECA by Western blot analysis as described in the Methods section. Glucose and glucose-6-phosphate results were identical, glucose-6-phosphate results are shown. Lane A, ECA Standard; Lane B, Medium C with 2% glycerol; Lane C, Glucose-6-phosphate; Lane D, Tunicamycin; Lane E, Tunicamycin and Glucose-6-phosphate.


A B C D E

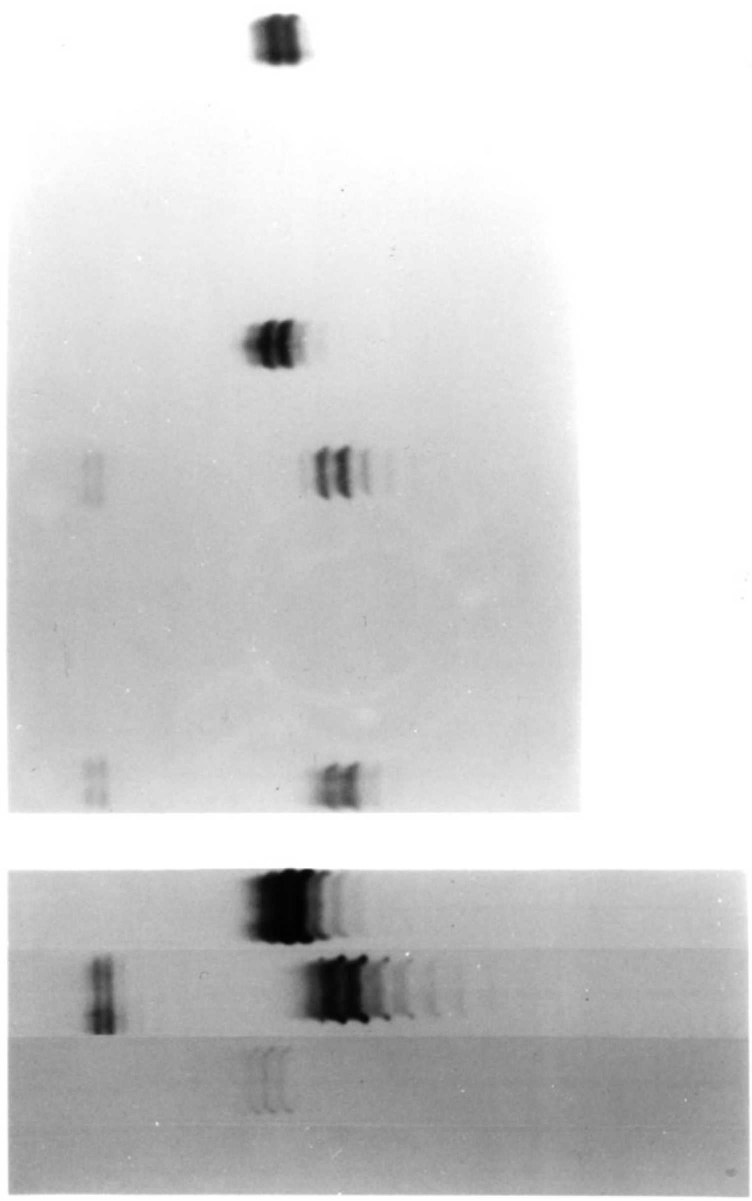
inhibit uptake of other carbohydrate sources including mannose. Glucose-6-phosphate does not have this effect. This established that the *rfe*-dependent synthesis of lipid I in this strain was sensitive to tunicamycin when grown in the absence of glucose.

Strain HR193 was grown overnight in medium C containing 2% glycerol. Five milliliters was then transferred to each of two flasks containing 50ml of fresh medium C and 2% glycerol. To one flask was added 0.5ml of a 1mg/ml tunicamycin solution (in 25<u>mM</u> NaOH) and the cultures were incubated at 37°C for 4-5 hours until an OD_{600nm} of 0.2 was reached. Due to the long incubation period required, an additional 500ul of tunicamycin solution was then added to the tunicamycin containing flask to "refresh" the antibiotic concentration and the incubation was continued for an additional 15 minutes. The two cultures were each subdivided into two 10ml subcultures, and one subculture of each set was supplemented with 50ul of 40% glucose-6-phosphate and 10uCi of 2[^{3}H]-mannose was added to all subcultures. The cultures were then incubated for one hour at 37°C. The bacteria were subsequently harvested and washed with 0.9% saline at 9,000xG. The washed cells were then resuspended in 0.5ml of electrophoresis sample buffer and analyzed for the presence of O8 side-chains by Western blot analysis.

The use of 2[^{3}H]-mannose in the O8 analysis provides an additional control for the detection system. The hydrogen in the 2-position is lost if mannose is catabolized and enters the general metabolism of the bacteria (Figure 3) (Fraenkel and Vinopal, 1973). Therefore, the presence of the label in the 2-position precludes labeling of anything but mannose-containing compounds. This may have proved important due to the leakiness (20% activity) of the *pmi* mutation moved into strain HR193 (Table 11).

Mannose-containing polymer bands were detected by SDS-PAGE after strain HR193 was grown in the presence of 2[^{3}H]-mannose (Figure 51). The "ladder-like" appearance of these bands is typical of a homologous series of polymers whereby the molecules in one band are one repeat unit smaller than the molecules comprising the next higher band. In addition, these bands "shifted" on the gel to a higher molecular weight when either glucose or glucose-6-phosphate was added during growth. It is believed that this is due to transfer of the O8 polymer to now completed LPS-core. However, tunicamycin treatment inhibited the formation of both sets of bands. Some O8 and ECA synthesis was observed when glucose or glucose-6-phosphate was added simultaneously with tunicamycin, demonstrating the importance of the order of addition of these compounds in the final protocol (Figure 51). The data in Figure 51 also demonstrate that the 2[^{3}H]-mannose containing polymer synthesized in the absence of glucose was labile to mild acid treatment, (0.1N HCl for 10 minutes at room temperature), whereas it was resistant to mild alkali treatment, (0.1N NaOH, 100°C, 10 minutes), (Yamamori et al., 1978). The mild acid lability supported the conclusion that this polymer was linked to an undecaprenyl carrier-lipid. However, the mild acid conditions would also release O8 side-chains from LPS by cleavage of the KDO linkages. In this case the LPS-"core" oligosaccharide would remain attached to the O8 side chains. The resistance of the glucose-minus O8 polymer to mild alkali suggested that the reducing sugar attached to the pyrophosphate does not contain a hydroxyl group in the 2-position. The presence of a hydroxyl group in the 2-position adjacent to pyrophosphate would lead to "cyclization" of the first phosphate to the hydroxyl group under alkaline conditions leading to cleavage between the two phosphates in the pyrophosphate linkage. However, treatment of the LPS-O8 form with

Figure 51. Determination of the Tunicamycin Sensitivity of O8 Synthesis. Strain HR193 (*manA, pgi*:Tn10) was grown overnight at 37°C in medium C containing 2% glycerol as the sole carbon source. Five-milliliters of the overnight culture was transferred to two flasks containing 50ml of fresh medium C supplemented with 2% glycerol. To one flask was added 0.5ml of a 1mg/ml tunicamycin solution (in 25mM NaOH), and the cultures were incubated at 37°C for 4-5 hours until an OD_{600nm} of 0.2 was reached. An additional 500ul of tunicamycin solution was then added to the tunicamycin containing flask and the cultures were incubated for an additional 15 minutes. The cultures were next subdivided to give two 10ml subcultures and to one of each set of the subcultures was added 50ul of 40% glucose-6-phosphate whereas all subcultures received 10uCi 2[^{3}H]-mannose. The cultures were then incubated for one hour at 37°C. The bacteria were then washed with 0.9% saline and resuspended in 0.5ml of electrophoresis sample buffer. The 2[^{3}H]-mannose labeled material produced from cells grown in Medium C containing 2% glycerol alone or supplemented with glucose-6-phosphate were furthered analyzed for acid and alkali lability. Aliquots of 50ul of the two samples were subjected to either 1N HCl for 20 minutes at room temperature (mild acid conditions), 1N HCL for 5 minutes at 100°C (strong acid conditions) or 0.1N NaOH for 20 minutes at room temperature (mild alkali conditions) All samples were prepared for electrophoresis and fluorography as described in the Methods section. Lane A, Tunicamycin; Lane B, Tunicamycin and Glucose-6-phosphate; Lane C, Medium C containing 2% glycerol; Lane D, Glucose-6-phosphate; Lane E, Medium C containing 2% glycerol; Lane F, mild acid; Lane G, strong acid; Lane H, mild alkali; Lane I, Glucose-6-phosphate; Lane J, Glucose-6-phosphate-mild acid; Lane K, Glucose-6-phosphate-strong acid; Lane L, Glucose-6-phosphate-mild alkali


A
B
C
D
E
F
G
H
I
J
K
L

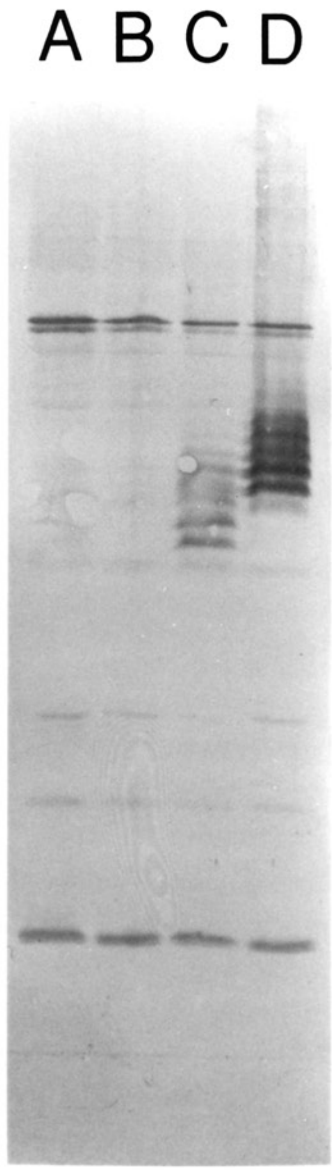
alkali would result in cleavage of the ester-linked fatty acids of Lipid A. It would be expected that this would result in a slight increase in mobility on SDS gels due to a reduction in molecular weight (Munford and Hall, 1986). This is in fact was observed when alkali treated LPS-linked material was analyzed by SDS-PAGE (Figure 51).

Figure 52 shows a Western blot using an anti-O8 polyclonal antibody for detection. The data from these experiments demonstrated that the bands observed by fluorography were indeed O8 side-chains.

The tunicamycin sensitivity of O8 synthesis, as well as previous data pertaining to the function of *rfe* (Meier-Dieter et al., 1992), strongly implicated a role for lipid I in O8 side-chain synthesis. Accordingly, in vitro experiments were performed in an attempt to demonstrate UDP-GlcNAc dependent synthesis of O8 polymers in the absence of UDP-glucose. These experiments utilized membrane preparations ("O8-membranes") similar to those described previously by Flemming et al. (Flemming and Jann, 1978). An alternative in vitro approach utilized cell envelope preparations, as described by Barr et al., for the in vitro synthesis of lipid II (Barr et al., 1988). More specifically, the "O8" membrane preparation was the 360,000xG total membrane pellet of Osborn's membrane separation procedure (Methods) and was resuspended in 400ul 0.1M Tris-HCl, pH7.5 (P360). The cell envelope preparation was the P160 preparation of Barr et al. (Barr et al., 1988) and is described in the "Methods" section.

Isolated [^{3}H]GlcNAc labeled-lipid I was prepared from strain SH5150 in the same manner as described in the "Methods" section for the determination of ECA intermediates. To the final lipid I preparation was added either 30ug of N-octylglucoside (Jann et al., 1985) or Mg^{+2}/EDTA (Behrens and Tabora, 1978), and the solvent was removed under a stream of

Figure 52. Determination of O8 Synthesis by Western Blot Analysis Using an Anti-O8 polyclonal Antibody. Strain HR193 (*manA*, *pgi* ::Tn10) was grown overnight at 37°C in medium C containing 2% glycerol as the sole carbon source. Five-milliliters of the overnight culture was transferred to a flask containing 50ml of fresh medium C supplemented with 2% glycerol and the culture was incubated at 37°C for 4-5 hours until an OD_{600nm} of 0.2 was reached. The culture was next subdivided to give four 10ml subcultures and to one of each three of the subcultures was added either 50ul of 40% glucose-6-phosphate, 50ul of 40% mannose or 50ul of both 40% glucose-6-phosphate and 40% mannose. All subcultures were then incubated for one hour at 37°C. The bacteria were harvested, washed and prepared for electrophoresis and Western blot analysis as described in the Methods section accept that anti-O8 polyclonal antibody diluted 1:1000 was substituted for the anti-ECA monoclonal antibody. Lane A, Medium C containing 2% glycerol; Lane B, Glucose-6-phosphate; Lane C, mannose; Lane D, glucose-6-phosphate and mannose


A B C D

nitrogen. Unlabeled GDP-mannose and the isolated lipid I were then incubated with the membrane preparations as subsequently described.

For the in-vitro "O8" reaction of Jann (Flemming and Jann, 1978); 110ul (55ug protein) of the P360 membranes were used to resuspend the N-octylglucoside-[^{3}H]lipid I preparation and 50ul was then mixed with 5ul 4mM GDP-mannose. The mixture was incubated at 37°C for 30 minutes, and the reaction was terminated with 2ul of 25% glycerol containing 5% SDS. The samples were then prepared for SDS-Page gel electrophoresis and fluorography as described in the "Methods" section.

For the in vitro "lipid II" reaction procedure of Barr et al. (Barr et al., 1988); the Mg^{+2}/EDTA-[^{3}H]lipid I preparation was gently resuspended by sonication in 41ul of 0.25M Tris-HCl, pH 8.2, 20.5ul 0.3M $MgCl_2$, 20.5ul 50mM 2-ME, and 20.5ul 0.1% triton X-100. Twenty-five microliters of the resuspended lipid I was mixed with 25-30ul (300ug protein) P160 envelopes, 5ul 4mM GDP-mannose, 15mM Tris-HCl, pH8.0, in a final volume of 60ul. The reactions were incubated for 30 minutes at 37°C and stopped by extracting with 1.0ml of chloroform/methanol (3:2, v/v). The extracts were treated in the same manner as described for ECA intermediates (Methods) and chromatographed on SG81 paper using solvent system B.

Lipid I dependent mannose incorporation into polymeric material could not be detected in either set of reactions.

The P160 preparation was also employed to examine UDP-GlcNAc- and UDP-glucose-dependent incorporation of [^{3}H]mannose into polymer from GDP-[^{3}H]mannose. These experiments depended on endogenous lipid carrier in the membrane preparation to serve as an acceptor. The reaction mixtures were of the same composition as before except that no lipid I was added and each reaction mixture contained contained 2.5ul/reaction of either 100uM

UDP-GlcNAc or 100uM UDP-glucose. In addition, tunicamycin at a final concentration of 10ug/ml was also added to some reactions before volume adjustment. The reactions were terminated by adding 0.5ml of water followed by centrifugation (25,000xg, 4°C). The pellets were then extracted three times with 1.0ml of chloroform/methanol (3:2, v/v) and two times with 1.0ml of chloroform/methanol/water (10:10:3, v/v/v), respectively. All extracts and the residue were saved and counted.

P160 membranes isolated from HR193 (O8^{+}) and HR191 (O8^{-} parent) were utilized in these assays. [^{3}H]Mannose incorporation in HR193-P160 membranes was observed to be thirty times greater than in the HR191 membranes. The incorporated counts in the HR193 membranes were located in the extract residue. No significant difference in counts was observed in any of the solvent extracts between the two strains. The incorporation was not UDP-GlcNAc or UDP-glucose dependent, nor was it inhibited by tunicamycin. An identical result was also observed using P160 envelope preparations obtained from strains 2443 (O8^{+}) and 2442 (O8^{-} parent). The in-vitro incorporation of [^{3}H]mannose from GDP-[^{3}H]mannose using P160 envelope preparations obtained from the two O8 strains, as well as the tunicamycin insensitivity of mannose incorporation, suggested that O8-polymer was being synthesized on preexisting lipid I.

These experiments failed to demonstrate the dependence of O8 polymer synthesis on lipid I or UDP-GlcNAc. However, the experiments also failed to demonstrate a UDP-glucose dependent incorporation of mannose. Therefore, it became necessary to isolate the lipid-linked O8 side-chains and identify the reducing terminal sugar. Strain HR193 was grown in 100 liters of medium C containing 2.0% glycerol and 0.1% beef extract (Difco) at 37°C in a 150-liter fermenter (New Brunswick) to an OD_{600nm} of 0.4. Fifty milliliters

of 40% mannose was added, and growth was allowed to continue for an additional hour; the cells were then harvested as described in the Methods. Enriched media such as medium A could not be used due to the presence of low levels of glucose which would have resulted in the synthesis of the LPS-linked O8 form. The final cell pellet was weighed and prepared as an acetone powder as described for lipid II and a dry-weight determined. Labeled preparations of HR193 were prepared by growing the strain in 25ml of medium C containing 2% glycerol and 0.1% beef extract in the presence of 10uCi of 2[^{3}H]mannose (23Ci/mmole) at 37°C to an OD_{600nm} of 0.6. The bacteria were harvested and converted to an acetone powder as described in the "Methods" section for the isolation of ECA-intermediates. The acetone powder was combined with the acetone powder obtained from the unlabeled fermenter grown cells. The combined pellet was resuspended in 5ml/gram dry weight of 10mM Tris-HCl, pH 7.5/10mM $MgCl_2$. Preliminary attempts to extract the resuspended acetone powder resulted in a viscous mass due to the presence of DNA. It was subsequently found that this problem could be overcome by the addition of lysozyme and DNase. Therefore, lysozyme (1mg/ml buffer) and DNase (1mg/gm pellet) were added and the mixture was incubated on ice for 2 hours. SDS was then added to a final concentration of 2% and the mixture was stirred for 30 minutes at room temperature. The suspension was centrifuged 10,000xg for 10 minutes at 25°C and the particulate fraction was discarded. Hydrochloric acid (4N) was added to the supernatant solution to give a final concentration of 0.2N. The acidified mixture was heated at 100°C for 10 minutes in a water bath cooled, and then centrifuged at 10,000xg for 10 minutes at 25°C. The supernatant was then lyophilized. To remove the SDS the residue was resuspended in 25ml of water and loaded onto a 2.5x15cm DEAE cellulose (chloride form) column

previously equilibrated with water. The column was eluted with water and 1 ml fractions were collected. The radiolabeled material was pooled, frozen and reduced to dryness under vacuum. The residue was resuspended in 25ml of 20mM Tris-HCl, pH 7.5/0.5M NaCl, and loaded onto a 1x13cm Con A-Sepharose 4B (Pharmacia) column that had been equilibrated with 20mM Tris-HCl, pH 7.5/0.5M NaCl. The column was washed with 40ml of the same buffer and then eluted with 50mM α-methyl-mannopyranoside in 20mM Tris-HCl, pH 7.5/0.5M NaCl. Fractions of 2ml were collected (Figure 53), and those fractions containing the radiolabeled material were pooled, frozen and reduced to dryness under vacuum. The residue was resuspended in 1 ml of water and loaded onto a 1x32cm Bio-Gel P2 column previously equilibrated with water. The column was eluted with water and fractions of 0.5ml were collected and those fractions containing the radiolabeled material were pooled. The total carbohydrate concentration of the preparation was determined using mannose as a standard. Isolation yields are shown in Table 12.

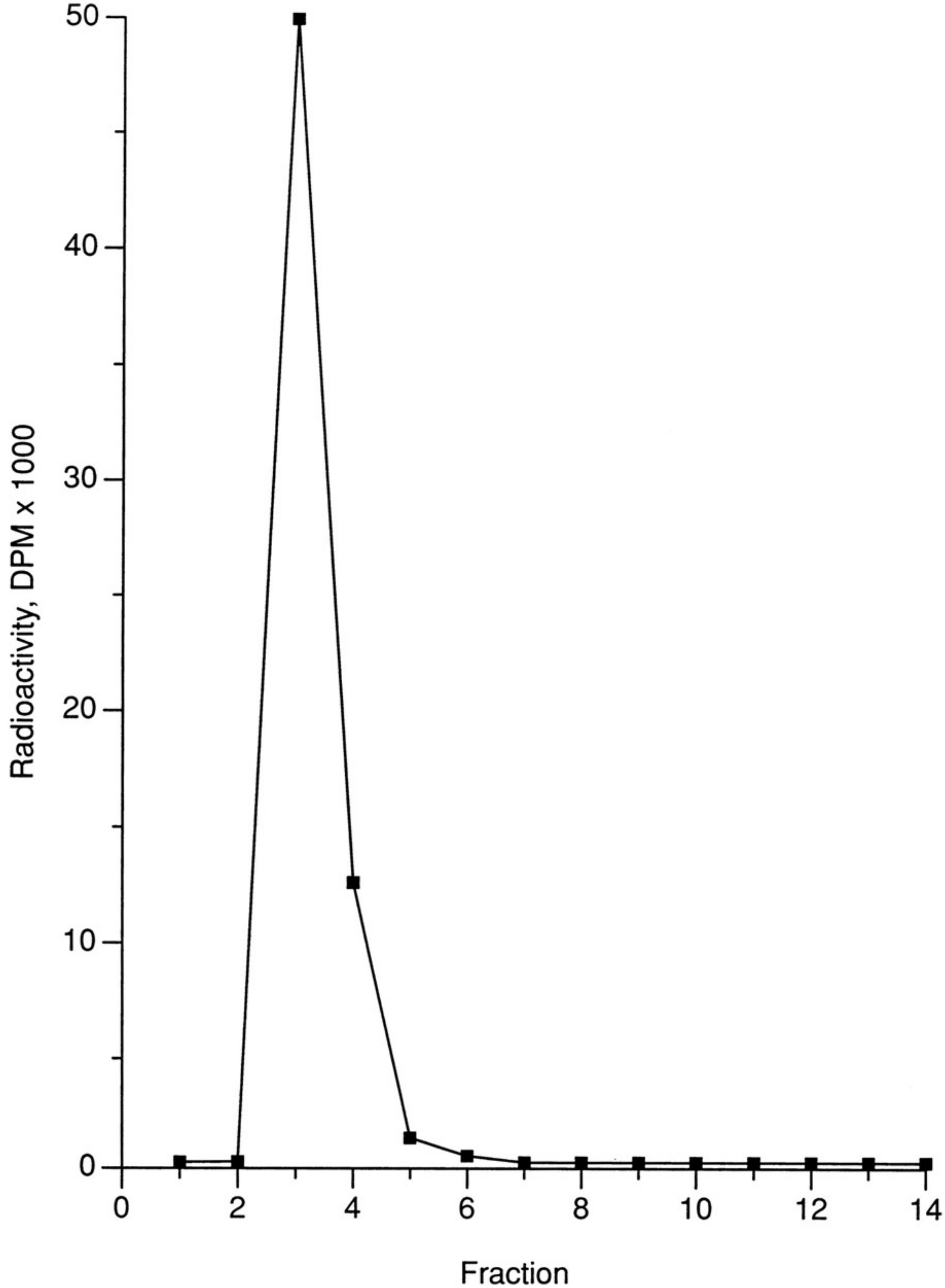
We were unable to extract lipid-linked O8 polymer into any of the organic solvent systems used in these studies, including 1-butanol which had been previously reported to solubilize lipid-linked O8 intermediates (Flemming and Jann, 1978). However, the mannose-containing material was extracted by 2% SDS, and this material became water soluble after mild acid hydrolysis. The addition of HCl precipitates a large amount of material, presumably protein. We found that addition of HCl to a final concentration of 0.2N was sufficient to overcome any neutralization of acid due to the precipitating material and lower the pH to 2. Following removal of the SDS by DEAE cellulose chromatography, the neutral fraction containing the mannose polymer was further purified by affinity chromatography using

Table 12. O8 Isolation Yeilds

Cell Wet Wt.	Cell Dry Wt.	ug. O8	Yield(dry wt.)
58.7gm	7.1gm	1.4mg	197ug/gm
88.9gm	11.4gm	5.4mg	474ug/gm

The O8 polymannan was isolated as specified in the Results section. The amount of O8 was determined by assaying total carbohydrate (Methods) in the final material using mannose as a standard.

Figure 53. Affinity Chromatography of O8 Polymer Using Sepharose-Linked Concanavalin A. The 2[^{3}H]mannose-labeled residue material in the "flow-through" fraction from DEAE cellulose chromatography (Results) was resuspended in 25ml of 20mM Tris-HCl, pH 7.5/0.5M NaCl, and loaded onto a 1x13cm Con A-Sepharose 4B (Pharmacia) column that had previously been equilibrated with 20mM Tris-HCl, pH 7.5/0.5M NaCl. The column was washed with 40ml of the same buffer and then eluted with 50mM a-methyl-mannopyranoside in 20mM Tris-HCl, pH 7.5/0.5M NaCl. Fractions of 2ml were collected and 50ul aliquots were counted.


50
40
30
20
10
0
Radioactivity, DPM x 1000
0
2
4
6
8
10
12
14
Fraction

Con A-Sepharose (Figure 53). The polymer was isolated after removal of α-Methyl-mannopyranoside by passage over BioGel P2. The purified material was then analyzed using the Dionex BioLC system. Prior to analysis part of the material was subjected to borohydride reduction, and both reduced and unreduced material underwent total acid hydrolysis followed by N-acetylation (Methods).

Mannose, GlcNAc, Glucose, mannitol, N-acetylglucosaminitol, and sorbitol were all separated by the Dionex BioLC gradient system (Figure 54). α-Methyl-mannopyranoside also gave a separate peak with a retention time of 2.62 minutes. The chromatogram of the unreduced O8 sample revealed a large mannose peak with a small GlcNAc peak (Figure 55). In contrast, the GlcNAc peak was not detected in the reduced sample; however, a peak corresponding to N-acetylglucosaminitol (retention time 3.03 minutes) was evident (Figure 56). Although there are several other peaks in both chromatograms, only the GlcNAc was affected by reduction. Addition of authentic N-acetylglucosaminitol to the reduced sample resulted in an increase in the signal at 3.03 minutes thus establishing the identity of the material as N-acetylglucosaminitol (Figure 57). Neither glucose or sorbitol were detected in the unreduced or reduced samples, respectively (Figure 58).

Figure 54. Separation of Monosaccharide Standards by High-Performance Anion-Exchange (HPAE) Chromatography. Standards of glucose, mannose, N-acetylglucosamine, sorbitol, mannitol and N-acetylglucosaminatol were dissolved in water to give a single solution containing each sugar at a final concentration of 1ug/ml. The mixture was analyzed by liquid chromatography with a Dionex BioLC liquid chromatograph equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Sugars were detected using a pulsed amperometric detector (PAD) as described in the Methods section. The monosaccharides were also chromatographed individually to establish retention times. Chromatography conditions were as follows: Eluant 1 was 300mM NaOH, Eluant 2 was not used, Eluant 3 was water, and Eluant 4 was 500mM sodium acetate. The concentratiopn of Eluant 3 was set at 100% at the start of chromatography. A gradient was produced by an increase in the concentrations of Eluants 1 and 4 to 1% and 2%, respectively, at 10 minutes followed by steps in the concentrations of Eluant 1 to 7% at 10.1 minutes and Eluant 4 gradient from 2%-10% during the 10.1 to 25 minute run period. Following each run, the system was flushed with Eluant 1, (33%) and Eluant 3 (67%) for 5 minutes and then equilibrated back to the starting conditions of 100% Eluant 3. The flow rate was 0.8ml/minute. Samples were injected using a 25ul sample loop. Due to the low initial concentrations of NaOH used in the gradient, 100mM sodium hydroxide was added post column at a flow rate of 0.5ml/minute to enhance the detection of the sugars by the PAD.

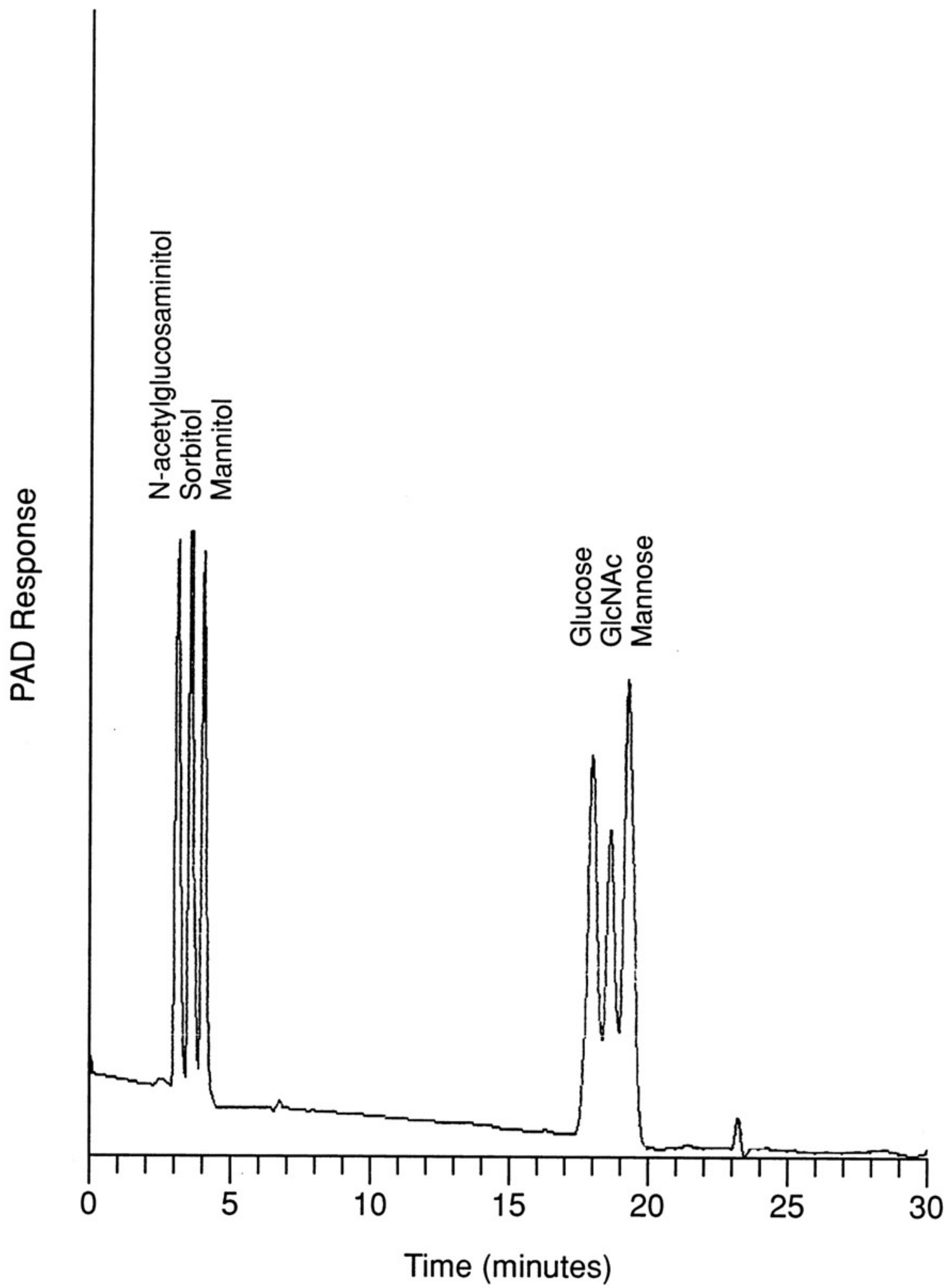

PAD Response
N-acetylglucosaminitol
Sorbitol
Mannitol
Glucose
GlcNAc
Mannose
0
5
10
15
20
25
30
Time (minutes)

Figure 55. Separation of Monosaccharides Released from Unreduced O8 Side-chains after Acid Hydrolysis by High-Performance (HPAE) Anion-Exchange Chromatography. Purified O8 polymer (approximately 1mg) was subjected to total acid hydrolysis and N-acetylation as described in the Methods section. The sample was resuspended to give a final concentration of 1ug/ml (based on 1mg starting material) in water. The solution was analyzed by liquid chromatography with a Dionex BioLC liquid chromatograph equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Sugars were detected by a pulsed amperometric detector (PAD) as described in the Methods section. Chromatographic conditions were as described in the Legend to Figure 54.

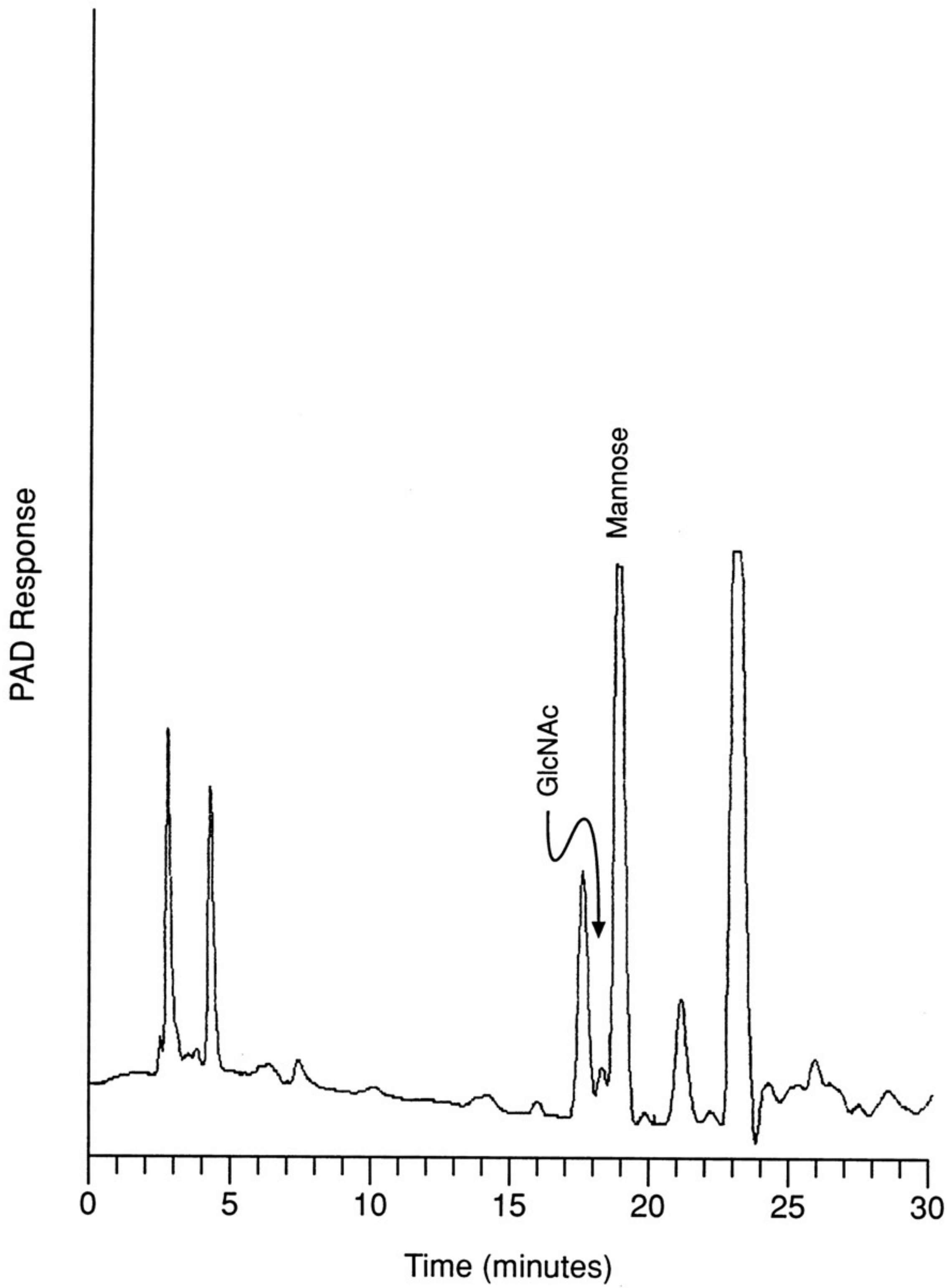
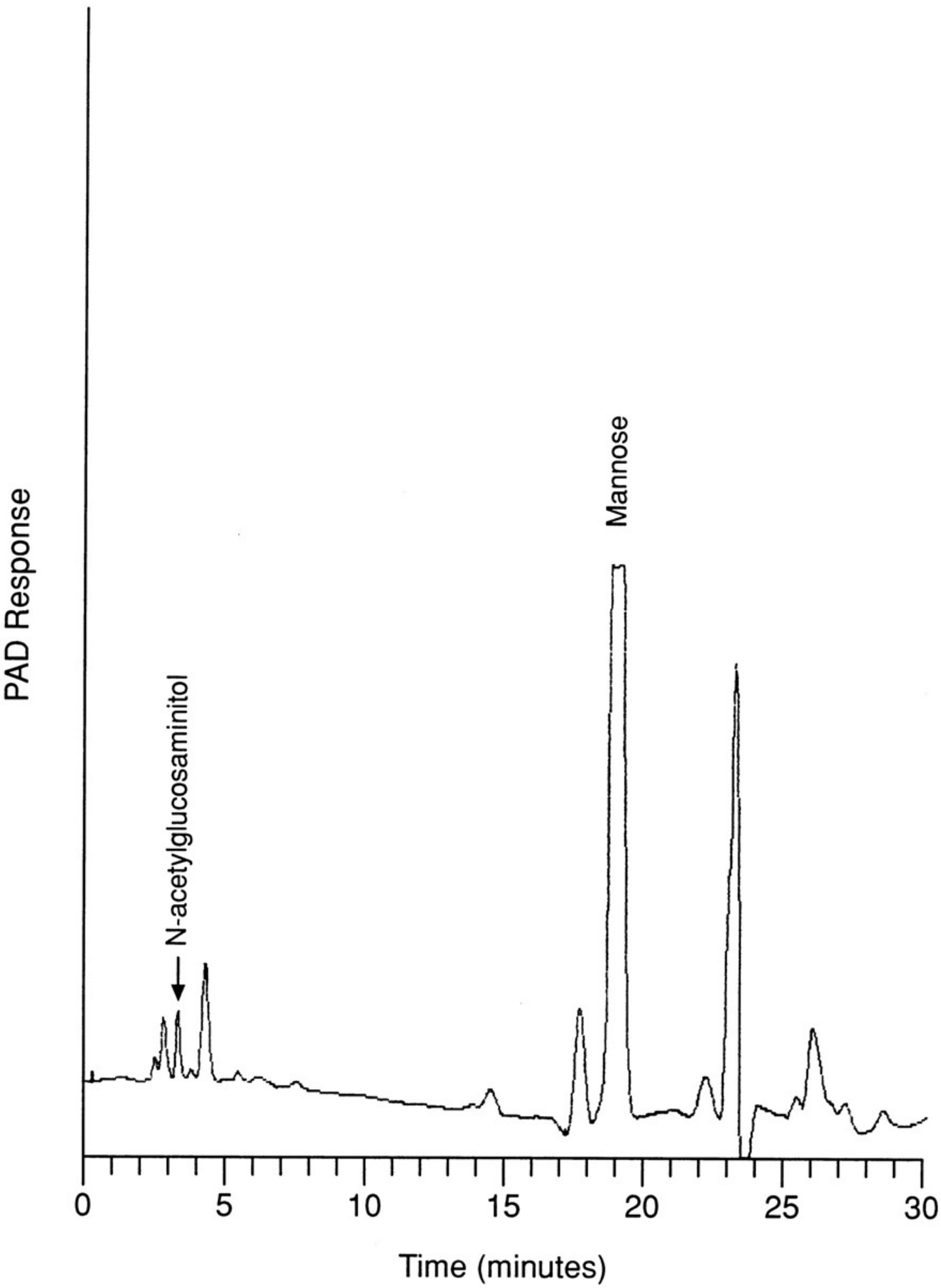

PAD Response
Mannose
GlcNAc
0
5
10
15
20
25
30
Time (minutes)

Figure 56. Separation of Monosaccharides Released from Reduced O8 Side-chains after Acid Hydrolysis by High-Performance Anion-Exchange (HPAE) Chromatography. Purified O8 polymer (approximately 1 mg) was subjected to sodium borohydride reduction followed by total acid hydrolysis and N-acetylation as described in the Methods section. The sample was resuspended to give a final concentration of approximately 1 ug/ml (based on 1 mg starting material) in water. The solution was analyzed by liquid chromatography with a Dionex BioLC liquid chromatograph equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250 mm). Sugars were detected by a pulsed amperometric detector (PAD) as described in the Methods section. Chromatographic conditions were as described in the Legend to Figure 54.



PAD Response
N-acetylglucosaminitol
Mannose
0
5
10
15
20
25
30
Time (minutes)

Figure 57. Identification of N-acetylglucosaminitol in Acid Hydrolysates of Reduced O8 Side-chains as Determined by High-Performance Anion-Exchange (HPAE) Chromatography.
N-acetylglucosaminitol standard solution (1ug/ml) was mixed 1:1 with a solution containing the sugars released from acid hydrolysis of reduced, O8 side chains (Figure 56). The resulting mixture was analyzed by liquid chromatography with a Dionex BioLC liquid chromatograph equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Sugars were detected by a pulsed amperometric detector (PAD) as described in the Methods section. Chromatographic conditions were as described in the Legend to Figure 54.

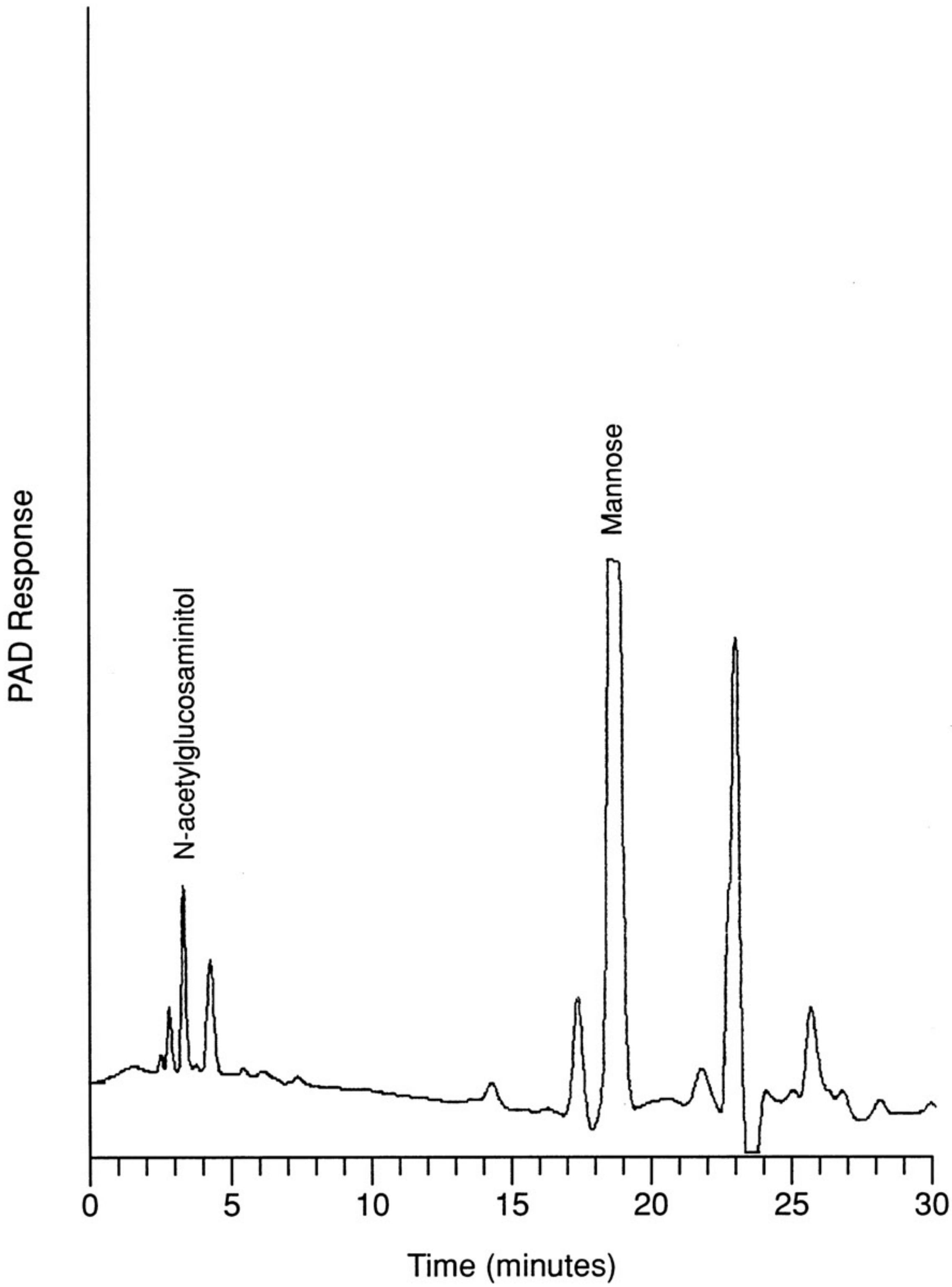
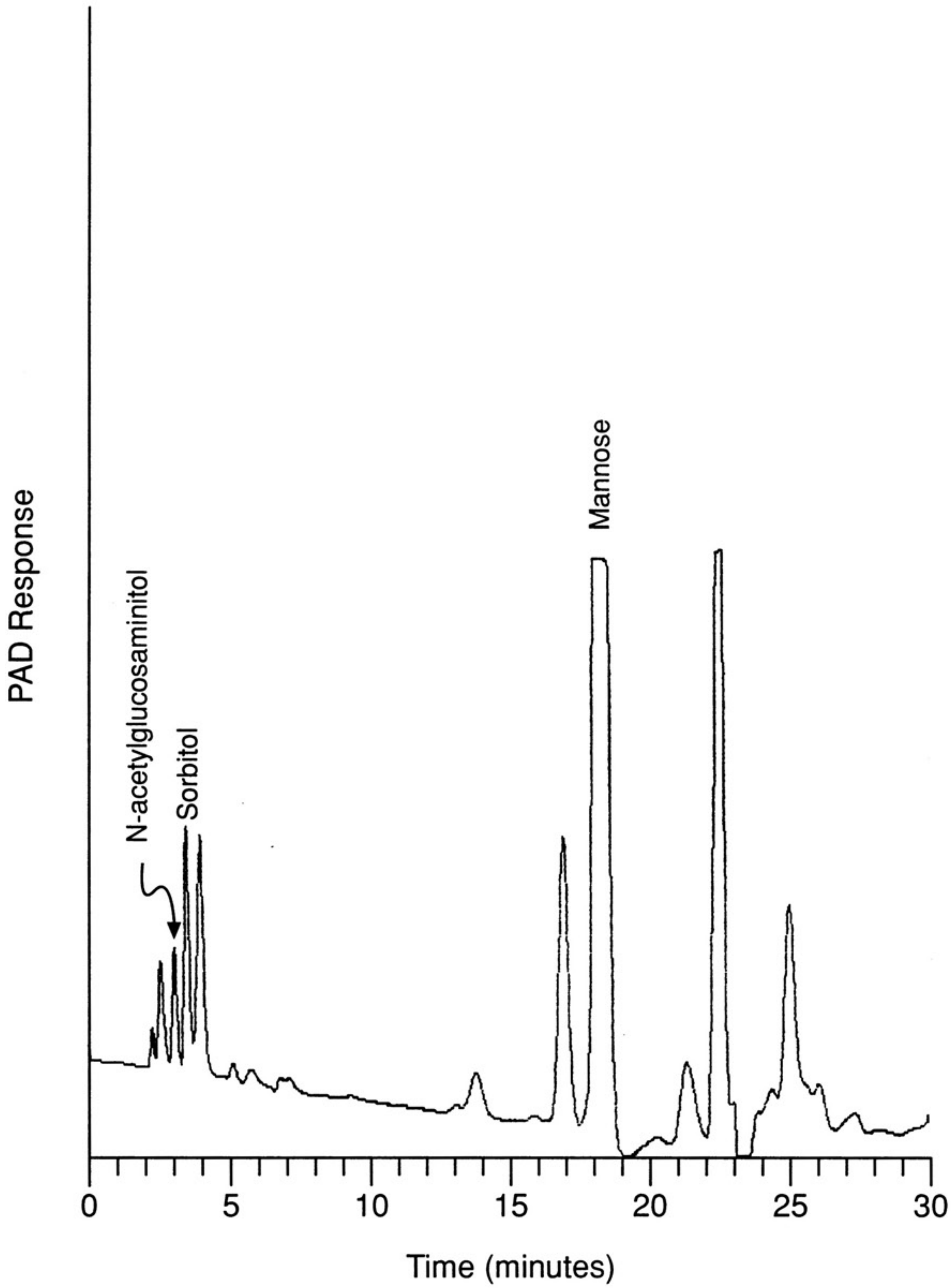

PAD Response
N-acetylglucosaminitol
Mannose
0
5
10
15
20
25
30
Time (minutes)

Figure 58. Absence of Sorbitol in Acid Hydrolysates of Reduced O8 Side-chains as Determined by High-Performance Anion-Exchange (HPAE) Chromatography. A sorbitol standard solution (1ug/ml) was mixed 1:1 with a solution containing the sugars released from acid hydrolysis of reduced, O8 side chains (Figure 56). The resulting mixture was analyzed by liquid chromatography with a Dionex BioLC liquid chromatograph equipped with a Dionex CarboPac PA-1 anion exchange column (4.6x250mm). Sugars were detected by a pulsed amperometric detector (PAD) as described in the Methods section. Chromatographic conditions were as described in the Legend to Figure 54.


PAD Response
N-acetylglucosaminitol
Sorbitol
Mannose
0
5
10
15
20
25
30
Time (minutes)

DISCUSSION

Lipid II Structure

The biosynthesis of many bacterial polysaccharides has been shown to involve lipid-linked intermediates. For most such pathways undecaprenol-phosphate has been shown to function as the acceptor of saccharide moieties in polymer synthesis including the assembly of peptidoglycan (Higashi et al., 1967, Higashi et al., 1970), O-antigen (Dankert et al., 1966), and teichuronic acid (Johnson et al., 1984). The results of early studies of *rfe*-gene function in ECA synthesis and assembly of the O side-chains of *Salmonella* group C_1 and L suggested the involvement of a lipid carrier molecule (Mäkelä and Mayer, 1974, Mäkelä et al., 1970). It was later shown that the earliest ECA intermediate, lipid I, comigrated with authentic GlcNAc-pyrophosphoryl-undecaprenol in several chromatography systems (Rick et al., 1985). With the identification of undecaprenol as the carrier lipid involved in the synthesis of lipid II (Figure 47), it now seems clear that ECA biosynthesis indeed requires this lipid carrier.

The structure of lipid II more clearly identifies the possible functions of the *rfe* gene. It now seems clear that the *rfe* gene does not code for a unique lipid carrier nor does it chemically modify the undecaprenol molecule as originally suggested by Mäkelä (Mäkelä and Mayer, 1974). Rather, it seems likely that *rfe* functions as the structural gene for the GlcNAc-1-phosphate transferase. This conclusion is supported by the results of

experiments which revealed that ECA synthesis in vivo was less sensitive to tunicamycin in strains possessing multi-copy plasmids containing the *rfe* gene. Furthermore, these studies also revealed a restoration of lipid I synthesis in *rfe* mutants following their transformation with such plasmids (Meier-Dieter et al., 1992). However, the lipid II structural information does not rule out the possibility that *rfe* is a "compartmentalization" factor; i.e., Rfe aids in maintaining the ECA enzymes together in a stable biosynthetic unit within a membrane site. An increase in the number of stable biosynthetic units would be the same as increasing a single enzyme if the "compartmentalization" factor was normally the limiting agent and the individual enzymes are inactive or unstable by themselves.

The combination of tandem mass spectrometry (MS/MS) with FAB-MS constitutes a powerful technique for the structural analysis of isoprenoid compounds (Kitaoka et al., 1990). An advantage of the FAB/MS/MS analytical technique over either conventional mass-spectroscopy techniques or FAB/MS alone is that the spectrum of a single compound can still be obtained from a mixture of compounds by analysis of the individual fragments obtained from the initial fragmentation of the parent compounds (Kitaoka et al., 1990). This proved to be particularly important in our studies for several reasons. First, we were able to determine the structure of lipid II from a mixture of several compounds which proved difficult to separate by conventional techniques. In fact, until the mass spectrum data was obtained (Figure 46), we believed that purified [^{3}H]-labeled preparations of lipid consisted of only a single compound based on two-dimensional TLC (Figure 43) and that purified unlabeled preparations contained at most two compounds. The compounds represented by the 1345, 1173 and 1145 molecular ions all seem to be related phospholipid-linked disaccharides. The

1345 species has been identified as lipid II. The 799 molecular ion, identified as PGP, was only observed in the unlabeled lipid II preparations, and it probably constitutes the second component observed in the two-dimensional TLC of this material. The isolation of this compound with lipid II may simply demonstrate the difficulty in the resolution of pertinent unlabeled fractions obtained by DEAE-cellulose chromatography using TLC and ashing as a means of detection as opposed to identifying pertinent fractions by measuring radioactivity. Perhaps of more interest are the structures of lipid IIa (M/Z=1145) (Figure 48) and lipid IIb (M/Z= 1173). These compounds have both been structurally characterized as ManAcA-GlcNAc-PP-diglycerides. The sn-1 and sn-2 positions of the glycerol are substituted with 16:0 and 16:1 fatty acyl substituents in lipid IIa, respectively. In contrast, the sn-1 and sn-2 positions are substituted with 16:0 and 18:1 fatty acyl groups in lipid IIb, respectively. These compounds may represent a new hitherto unidentified class of compounds for procaryotes. There are several possible explanations for the occurrence of lipids IIa and IIb. The first possibility -and least interesting- is that the synthesis of these compounds simply reflects a mechanism for "ECA-trace" strains to free up undecaprenol phosphate for more critical functions such as peptidoglycan synthesis. An estimate, calculated from the ^{3}H-lipid II yields of this work (Table 10) and a cell dry weight value of 2.8 X10^{-13} (Neidhardt, 1987) put the total number of undecaprenol phosphate molecules as lipid II in these strains at approximately 150. There is a limited amount of undecaprenol phosphate available in the cell and therefore the ability to reutilize this lipid is critical (Park, 1987). This is, in fact, the basis for the effectiveness of the antibiotic Bacitracin which blocks the regeneration of undecaprenol phosphate in peptidoglycan synthesis. A more interesting

possibility is that these lipids might represent the next step in ECA synthesis prior to Fuc4NAc transfer from TDP-Fuc4NAc and polymerization (Figure 4). This would be a departure from the mechanisms of biosynthesis of other polysaccharides (Rick 1987) where polymerization occurs at the level of undecaprenyl-linked intermediates. These compounds could also be artifacts of the lipid II accumulation. More specifically, high concentrations of lipid II could possibly overwhelm the final transfer reaction that normally transfers polymer to the appropriate final acceptor. This also assumes that the structure of the phospholipid form of ECA has been incorrectly identified (Kuhn et al., 1988) and is instead linked to a diglyceride via pyrophosphate. In either of the latter two proposals, the transfer to "phospholipid" could presumably be a step toward translocation across a membrane or transfer from the inner to the outer membrane as isoprenoid-linked polymers seem to confined to the cytoplasmic face of the inner membrane. The pyrophosphate linkage of such intermediates would in turn provide energy for transfer of the polymer (or dissaccharide in this case) to some other structure (Dankert et al., 1966) such as LPS or another lipid. The observation of larger amounts of lipid IIa containing the 16:1 fatty acid in the C-2 position relative to lipid IIb with a 18:1 fatty acid in the C-2 position is consistent with the previously reported fatty acid composition of ECA-PL forms (Kuhn et al., 1988), and it is also consistent with the overall fatty acid composition of cell envelope phospholipids (Shaw and Ingraham, 1965). The pyrophosphorylipid structure of ECA-PL may also explain the occurrence of "cyclic" ECA observed in the periplasm of *Shigella sonneii* phase I cells (Dell et al., 1984). The pyrophosphate linked lipid might act as a donor in a reaction in which the reducing terminal sugar is transferred to the non-

reducing terminal sugar resulting in formation of a cyclic artifact in the periplasm.

Synthesis of "Trace" ECA in Δ*rfb* Mutants

There have been several explanations for the synthesis of trace amounts of ECA in Δ*his-rfb* (trace) mutants of *S. typhimurium*. The suggestion that a strict lack of specificity of UDP-glucose pyrophosphorylase might account for small amounts of TDP-glucose synthesis in mutants lacking TDP-glucose pyrophosphorylase activity (Lew et al., 1986) could not be substantiated in this study. This explanation also ignores the fact that Δ*his-rfb* mutants also lack TDP-glucose oxidoreductase activity. Accordingly, the movement of a *gal*U mutation into the "trace" strain (Figure 17) demonstrated that UDP-glucose pyrophosphorylase was not involved in ECA synthesis. This result indicated that either there is another enzyme with TDP-glucose and/or UDP-glucose pyrophosphorylase activity or, alternatively, there exists a mechanism for synthesis of an ECA-like polymer which is structurally distinct from ECA but which nevertheless reacts with the anti-ECA monoclonal antibody. Thus, substitution of Fuc4NAc with another sugar to form a new trisaccharide unit might account for the synthesis of "trace" ECA. In addition, the synthesis of a polymer consisting of only ManAcA-GlcNAc repeat units also seemed possible. The latter two possibilities seem unlikely since "ECA-trace" is detected by a monoclonal antibody which is assumed to be very specific even though ManAcA is the epitope on the ECA molecule that is recognized by the antibody (Kuhn et al., 1978). However, recent work suggests that the monoclonal antibody 898 may also recognize ManNAcA in ECA precursors (Acker and Kammerer,

1990). If this observation is correct, it would allow for the possibility of detecting an altered ECA polymer. In addition, the movement of a *pgi* mutation into the ECA-trace background demonstrated that trace-ECA was a glucose dependent polymer, since ECA-trace synthesis was abolished in these mutants (Figure 20). The synthesis of UDP-GlcNAc and UDP-ManNAcA are not dependent on the ability of the cell to synthesize glucose (Figure 3). Therefore, the trace-polymer still contained a glucose-derived sugar, but this data gave no information as to the identity of the third sugar. However, the ECA-minus phenotype of the *rff*T construction (Figure 28), even though not in the "trace" background, suggests that this sugar is Fuc4NAc and that "ECA-trace" has the same structure as wild-type ECA.

A review of the literature suggests the strong possibility that *S. typhimurium* may contain duplicate copies of the structural genes encoding TDP-glucose pyrophosphorylase and TDP-glucose oxidoreductase in the *rff* region. Thus, "ECA-trace" was produced in strains of *Salmonella* type C1 when the *rfe-rff* region of these strains was replaced with the *rfe-rff* region from *S. typhimurium* (type B). The C1 O-antigen does not contain any TDP-glucose derived sugars (Figure 3). In addition, replacement of the type B *rfb* region with the type C1 *rfb* genes in *S. typhimurium* also resulted in an ECA-trace phenotype (Mäkelä and Mayer, 1974). Furthermore, the replacement of the *rfe-rff* region of *S. typhimurium* Δ*his-rfb* mutants with either the *rfe-rff* region of *Salmonella* type C1 (Lew et al., 1986) or *E. coli* (Schmidt et al., 1976) resulted in synthesis of normal amounts of ECA in the recipient strain. It was concluded from these experiments that, with the exception of *S. typhimurium*, all other Enterobacteriaceae strains tested have fully expressed genes for TDP-glucose pyrophosphorylase and TDP-glucose oxidoreductase in the *rff* region. The observation of the ECA-trace

phenotype in group C1 *Salmonella* by introduction of the *S. typhimurium* (group B) *rff* region suggests that the two genes may also be present in the group B *rff* region, but are expressed at reduced levels. This hypothesis is further supported by a *S. typhimurium* strain that has the *rfb* region totally deleted and also has a mutation in *gal*U (Nakae and Nikaido, 1971a). Strain, HN300 still has a small but measurable TDP-glucose pyrophosphorylase activity. Unfortunately, this strain has not been tested for ECA, and it was unavailable for us to test. In addition, multiple forms of TDP-glucose oxidoreductase have been observed in *S. typhimurium* (Nikaido et al., 1967) lending further support to the gene duplication hypothesis. Low activity of TDP-glucose oxidoreductase can be detected in strain HN300 and other deleted *rfb* strains, and it was suggested that this activity is due to a structural gene located outside the *rfb* region (Nakae and Nikaido., 1971a, Nikaido et al., 1967).

The availability of the *rfbA* and *rfbB* genes on the pPR645 plasmid make it possible to determine if additional copies of these genes are present in *S. typhimurium* by developing probes for Southern blot analysis. Further if such copies were discovered, these same probes could be used in Northern blot analysis to determine the level of transcription of the duplicate genes. The availability of the *rfe* gene on pCA53 (Meier-Dieter et al., 1992) would enable a determination of linkage of these gene copies to the *rfe-rff* region.

Role of Lipid II in the Altered Permeability of ECA-Trace Mutants

The phenotypic characterization of the *rff*T construction HR210 indicates that the sensitivity of this mutant to SDS (and other hydrophobic agents) is associated with the lipid II accumulation. The possibility of genes

in the deleted portion of the *his-rfb* region of the original ECA-trace strains having any involvement in the SDS sensitivity phenotype would seem to be eliminated, as the parent strain SA3858 is wild type with respect to the *rfb* region. In addition, the *rfb* complementation studies of this work (Figure 32) indicate that only genes involved in TDP-Fuc4NAc synthesis were associated with the sensitivity of ECA-trace strains to hydrophobic agents. As described previously, movement of the group C *rfe-rff* region into *S. typhimurium* Δ*his-rfb* mutants yielded SDS-resistant phenotypes (Mäkelä et al., 1976, Lew et al., 1986). This provided further support for a lack of involvement of genes in the *his-rfb* region in the hydrophobic sensitivity phenotype.

Membrane localization experiments revealed that lipid II is associated with the inner membrane (Figure 6). However, the SDS lysis experiments indicated that spheroplasts prepared from ECA-trace strains were no more sensitive to SDS then spheroplasts obtained from wild-type strains (Figure 10). In addition, it was observed that intact ECA-trace strains were more SDS sensitive (Figure 9), and they were also RNase leaky. Accordingly, these observations suggest that the accumulation of Lipid II in the inner membrane is accompanied by a defect in the outer membrane. The question then becomes how does the inner membrane accumulation of lipid II cause a defect in the outer membrane? A comparison of the data collected for the ECA-trace strain to other strains sensitive to hydrophobic agents may provide clues. The MIC data of this study indicates that the ECA-trace strain has hydrophobic sensitivity patterns (with respect to both the types of agents and their inhibitory concentrations) very similar to those of deep rough LPS strains with Re- and Rd_1- chemotypes (Tables 5-7). This is in contrast to the qualitative data previously reported for the sensitivity of ECA-trace and Re-chemotype strains to antibiotics and other agents (Mäkelä

et al., 1976). These investigations reported that the ECA-trace strain was resistant to polymyxin at a concentration of 0.3 IU/ml while an Re-chemotype strain was sensitive to the same concentration. The ECA-trace strain was also reported to be resistant to gentian violet (crystal violet) and erythromycin while the Re-chemotype strain was sensitive. The current data shows that the ECA-trace strain is actually more sensitive to polymyxin B than the Re-chemotype strain, and it is also quite sensitive to crystal violet and erythromycin (Table 6). These contradictory results may be due to several factors. The qualitative experiments of Mäkelä et al. were performed by spreading the strains over nutrient agar plates containing each agent at specific concentrations (Mäkelä et al., 1976). The effectiveness of the agent could be compromised by other components in the medium (Washington and Sutter, 1980). It has also been observed that the ECA-trace strains give rise to mutants that are resistant to many of the hydrophobic agents with high frequency. If the revertant population was particularly large, it could lead to an interpretation of resistance in the plate assay. In addition, the amount of bacterial inoculum in these plate assays was not rigorously controlled. In contrast, although the microdilution assays reported here were also subject to media interference and and emergence of resistant mutants, the results were reproducible and the inoculum was carefully controlled.

A notable difference between ECA-trace and Re-chemotype strains may be their differential sensitivity to polymyxin B (Table 6). The data of this study clearly suggests that ECA-trace strains are more sensitive to this antibiotic. Polymyxin has been reported to disrupt LPS-LPS interactions due to its multiple positive charges by displacing or substituting for divalent cations and/or natural polyamines in the outer membrane (Sukupolvi and Vaara, 1989). Therefore, the polymyxin B sensitivity of the ECA-trace strains

may be due to the observed "mixed" LPS chemotypes (Figure 34). The mixed LPS forms could have weak interactions with each other due the differences in charge as a result of the variable amount of charged sugars, phosphate and ethanolamine present. This "weak" LPS-LPS interaction would presumably render polymyxin more effective in these strains. However, in the presence of EDTA, the ECA-trace strain was no more sensitive then it's parent strain SH5150 to either SDS or lysozyme in the lysis experiments (Figures 12, and 14). This would indicate that "mixed" LPS interactions may not be involved in the polymyxin sensitivity. The EDTA concentration employed in these studies may not have been sufficient to allow differentiation of strains possessing different LPS compositions. There might also be some as yet unidentified factor or factors that may be present (or lacking) in the outer membrane of the ECA-trace strain which increases its susceptibility to polymyxin. Nevertheless, the cytoplasmic membrane is believed to be the ultimate site of action for polymyxin (Nikaido and Vaara, 1985). Thus, it seems likely that ECA-trace strains possess an outer membrane defect that simply allows easier access of this agent to the inner membrane.

The ECA-trace strain was equally sensitive to anionic (SDS), cationic (MBE) and nonionic (TritonX 100) detergents (Table 7). Furthermore, the degree of hydrophobicity of these agents and not their charge seemed to be the more important factor in determining their effectiveness. The ECA-trace strain's resistance to most of these same agents when grown on minimal media (Figure 21) was rather surprising. Although the basis for this observation is not understood, these data may reflect a structural reorganization of the outer membrane that is in some way brought about by growth in minimal media. Strains of *E. coli* possessing an *env*A mutation

have been shown to undergo a reversal of hydrophobic sensitivity when their outer membrane protein content was increased (Grundstrom et al., 1980). A similar phenomena may be occurring in ECA-trace strains as a result of growth in minimal media. The sensitivity of the ECA-trace strain to SDS in medium A supplemented with 2mM $MgSO_4$ and/or 0.1mM $CaCl_2$ were performed to determine if divalent cations had provided the protective effect in the minimal media. The trace strain remained SDS sensitive indicating that calcium and magnesium were not involved in the protective effect.

The tentative identification of a "mixed" LPS chemotype for the ECA-trace strain (Figure 34) provides the only tangible correlation to other mutants known to be sensitive to hydrophobic agents. The *nbs*B mutation of *E. coli* (Coleman and Leive, 1979) and the *rfa*H mutation in *S. typhimurium* (Lindberg and Hellerqvist, 1980) both produce mixed LPS chemotypes-Ra/Re equivalents for the *nbs*B mutant and Ra/Rc for the *rfa*H mutant. The *rfa*H gene is a possible regulator for many of the glycosyltransferase genes involved in LPS synthesis. Lipid II may have a similar effect by directly interfering with the enzymatic function of these transferases in the inner membrane.

The identification of lipids IIa and IIb (Figure 48) and the apparent localization of ECA to the inner membrane (Figure 8) raises other possibilities as to the nature of the outer membrane defect in ECA-trace strains. The location of ECA in the inner membrane was unexpected, since previous work by other investigators indicated that ECA was an outer membrane component (Kuhn et al., 1988). Early studies directed at determining the localization of ECA involved the use of heterologous ECA-antisera and immunofluorescent techniques on whole cells (Aoki et al., 1966), and they

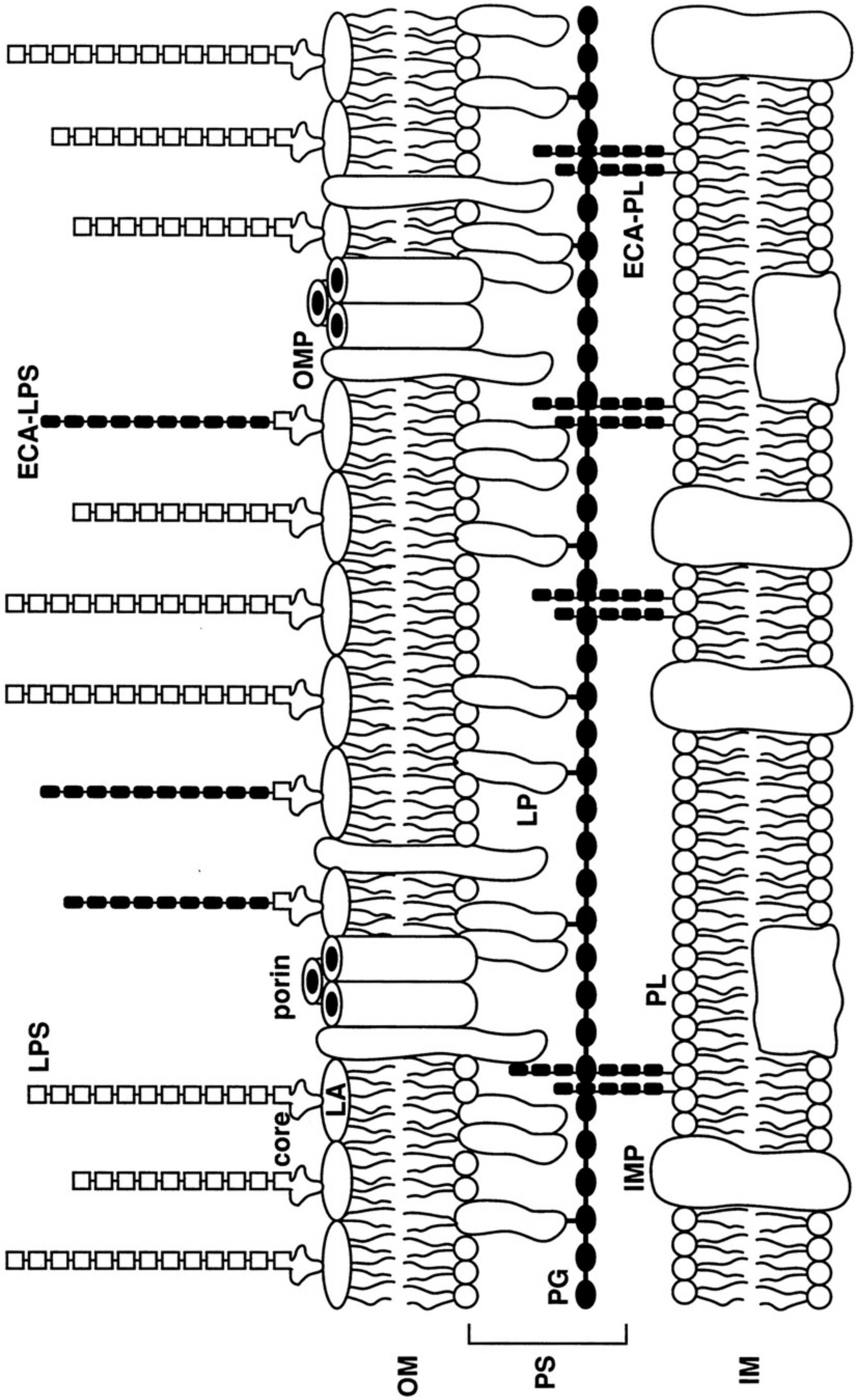
showed that ECA was associated with the cell envelope. Johnson and co-workers fractionated *E. coli* and *S. typhimurium* cells first by differential centrifugation (Domingue and Johnson, 1974) and later by sucrose density gradient centrifugation (Johnson et al., 1976); those investigators came to the conclusion that ECA was present in both the inner and outer membrane as determined by hemagglutination inhibition using heterologous anti-ECA-antisera for detection. They also observed that the ECA of *S. typhimurium* was more "loosely" associated to the cell than was the case with *E. coli* (Johnson et al., 1976). In another study, *E. coli* strains with both the immunogenic (ECA_{LPS}) and haptenic (ECA_{PL}) ECA forms and strains with the haptenic ECA form only, were labeled with preabsorbed heterologous ECA-antisera labeled with ferritin (Rinno et al., 1980). The immunogenic ECA strains showed prominent ferritin deposition in the outer membrane area. This would be expected since the ECA was linked to LPS. The haptenic ECA strain labeled weakly, and only in distinct areas of the outer membrane. Similar results were obtained using immunofluorescence in the same study (Rinno et al., 1980). *Proteus mirabilis* L forms were used as a natural model to test ECA localization (Rinno et al., 1980a). Wild-type, intermediate L forms and a stable L form of *Proteus mirabilis* were labeled using preabsorbed heterologous anti-ECA-antisera followed by either ferritin-conjugated anti-IgG or fluoresceine-labeled anti-IgG. In these experiments it was shown that ECA disappeared along with all outer membrane and cell wall components. It was concluded that since the stable L form was ECA-minus, ECA must be associated with the outer membrane. However, these observations may simply reflect the fact that ECA, like other components of the cell envelope (e.g., LPS, OMPs, peptidoglycan, ect.,) is no longer required in the L form. Other studies using the same immunological techniques and strains

possessing a rough-LPS also reported that ECA was in the outer membrane (Acker et al., 1992). More recent reports using immunogold labeling with anti-ECA monoclonal antibody 898 on *E. coli* rough strains have revealed that ECA and/or ECA precursors are located throughout the cell (Acker et al., 1986, Acker and Kammerer, 1990). Indeed these studies suggest an apparent association of ECA with ribosomes.

Until further studies are done to corroborate the results of this study, we must acknowledge the possibility that our observation that ECA is associated with the inner membrane may be an artifact. However, the published information to date can be misleading. Many of the strains investigated in previous studies contained the immunogenic (ECA_{LPS}) form of ECA which would certainly be localized in the outer membrane. *S. typhimurium* does not synthesize the immunogenic ECA form. It has been reported that strains containing only the non-immunogenic (ECA_{PL}) form of ECA react poorly in immunoreactivity assays. Accordingly, it has been suggested that ECA_{PL} is located in an inaccessible site (Meier-Dieter et al., 1989). Many of the strains used in the published ECA localization studies were "rough" strains or strains of uncertain outer membrane integrity. Therefore, the access of ECA to detection agents or the stability of the ECA at a particular subcellular site during preparation is in question. However, our observations are perhaps more meaningful than those of previous studies, particularly those of Acker et al., since we used monoclonal anti-ECA antibody in conjunction with Western blot analysis to detect ECA in membrane fractions (Figure 8). The manipulations involved in the Western blot analysis should have eliminated any ECA precursors or, at the very least, these precursors would have been observed as separate bands on the Western blot. No such bands were observed.

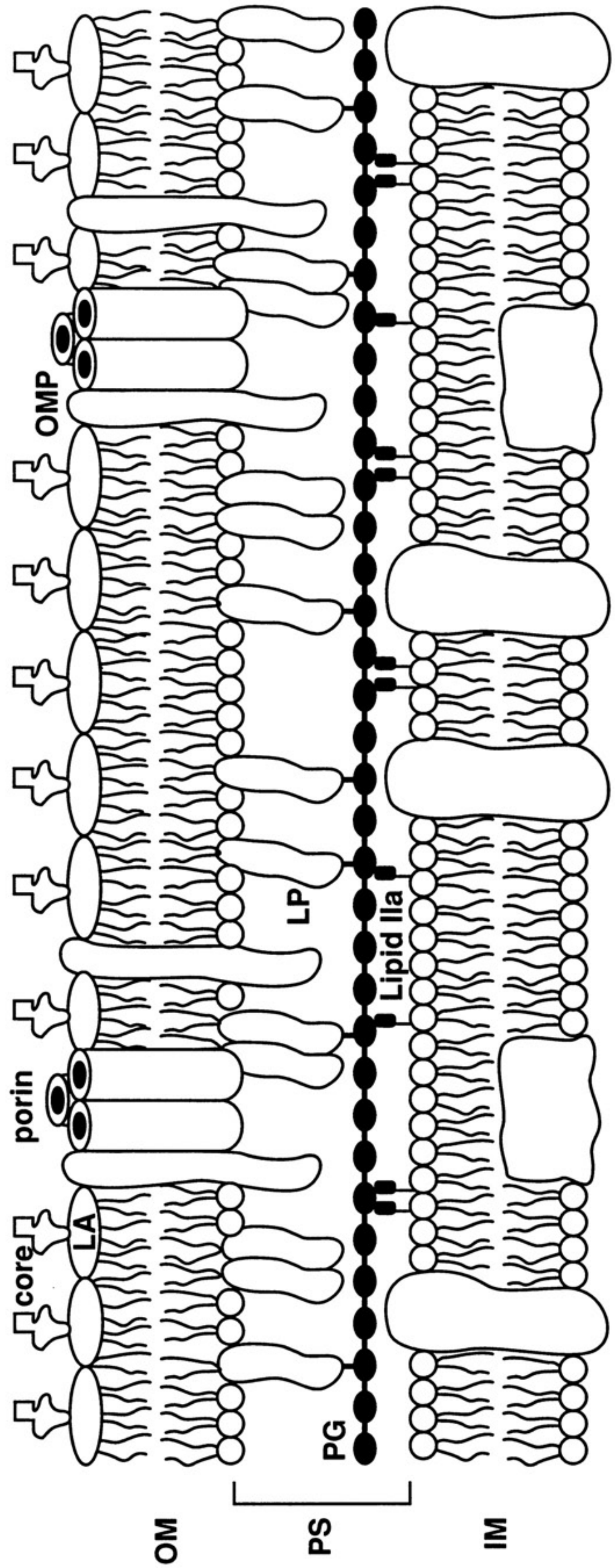
Based on our observations with *S. typhimurium*, we conclude that ECA_{PL} is indeed localized in the inner membrane. There could be several functions for ECA on the periplasmic face of the inner membrane. One function might be that ECA_{PL} serves as a structural link from the inner membrane to the peptidoglycan layer in a similar manner as lipoprotein functions as a link between the peptidoglycan layer and the outer membrane. A revised Enterobacteriaceae cell envelope model reflecting this possibility is shown in Figure 59. It is presumed that if such a cross-link occurs it would be through one or more of the ManAcA residues of the ECA polysaccharide chain to the muramic acid residues of the peptidoglycan. Precedent for this comes from the association of teichoic and teichuronic acids of the gram-positive bacteria with peptidoglycan. Teichoic and teichuronic acids are carbohydrate polymers consisting of glycerol-phosphate,ribitol-phosphate, or other sugar-phosphate residues (Ward, 1981). These polymers possess phospholipid aglycones which serve to anchor them to the outer surface of the membrane. There also exist "wall" forms which are covalently linked to some of the muramic acid residues of the peptidoglycan layer (Van Driel et al., 1971; Ward, 1981). This then raises another possibility to explain why ECA-trace strains are sensitive to hydrophobic agents. If ECA_{PL} functions as the inner membrane to peptidoglycan link, then the presence of lipid IIa (and lipid IIb) could cause an alteration in the structure of the link. Thus, substitution of lipid IIa(b) links for wild-type ECA_{PL} would lead to a shortening of the normal distance between the cell envelope layers to as little as 2% of the original width of the periplasmic space, based on a normal ECA-length of 40 trissacharide units, reduced to the single dissacharide present on lipids IIa and IIb (Figure 60). The decrease in distance could lead to inner and outer membrane layer mixing due to the

Figure 59. Revised Structure of the Cell Envelope of Gram-Negative Bacteria. The apposing figure depicts a proposed schematic representation of the structure of the cell envelope based on the ECA localization data obtained in this study. Note that the location of Phospholipid-bound ECA on the periplasmic face of the inner membrane is in contrast to the location of this molecule as shown in Figure 1. Also shown is a proposed "linkage" to the peptidoglycan layer. Abbreviations: OM, outer membrane; IM, inner membrane; PS, periplasmic space; PG, peptidoglycan (murein); IMP, inner membrane protein; PL, phospholipid; LP, lipoprotein (note bound and free forms); OMP, outer membrane protein including porins; LPS, lipopolysaccharide (O-antigen oligosaccharide repeat units are represented as open boxes); LA, lipid A; ECA-PL, phospholipid bound enterobacterial common antigen (haptenic form); ECA-LPS, lipopolysaccharide bound enterobacterial common antigen (immunogenic form). ECA oligosaccharide repeat units are represented with solid ovals. (Adapted from Lugtenberg, B., and L. van Alphen. 1983.)



LPS
ECA-LPS
core
LA
porin
OMP
LP
PG
ECA-PL
PL
IMP
OM
PS
IM

Figure 60. <u>Salmonella</u> <u>typhimurium</u> ECA-trace Strain, Cell Envelope Model. The apposing figure depicts a proposed schematic representation of the structure of the cell envelope of a *Salmonella typhimurium* ECA-trace Strain based on the location of ECA and lipid IIa as determined in this study. Note the reduction of the periplasmic space and the close proximity of inner and outer membranes. The O-side chains are absent from the LPS due to the deletions in rfb in these mutants. Abbreviations: OM, outer membrane; IM, inner membrane; PS, periplasmic space; PG, peptidoglycan (murein); IMP, inner membrane protein; PL, phospholipid; LP, lipoprotein (note bound and free forms); OMP, outer membrane protein including porins; LA, lipid A.


core
LA
porin
OMP
LP
Lipid IIa
PG
OM
PS
IM

close proximity of the two membranes. This seems unlikely as we observed normal membrane separations with the ECA-trace strain (Figure 5). If inner and outer membrane mixing were occurring, a larger "middle" membrane band would have been expected in the separation profile (Osborn et al., 1972).

Bayer junction sites are areas of the cell envelope where the inner and outer membrane are fused together (Bayer, 1979; Bayer et al., 1982). It has been suggested that these sites play a crucial role in outer membrane assembly, in part acting as a "conduit" for translocation of outer membrane components (Bayer, 1979; Bayer et al., 1982; Nikaido and Vaara, 1985). The shortening of the normal distance between the cell envelope layers due to lipids IIa and IIb would lead to a shortening of the Bayer junction sites. This shortening might lead to premature transfer of outer membrane components to the outer membrane before their biosynthesis is complete. The "mixed" LPS chemotype of the ECA-trace strain would be consistent with this model. A combination of incomplete outer membrane components and possibly increased phospholipid transfer to the outer membrane could be the factors that lead to the loss in barrier function and hydrophobic sensitivities observed in the ECA-trace strain.

This hypothesis could be tested by analysis of the ECA-trace strain by electron-microscopy with attention to the structure of the cell envelope and careful measurement of the width of the periplasmic space in electron-micrographs of carefully made cross sections of wild-type, ECA-trace and ECA-negative strain cells. A quantitative and qualitative analysis of the composition of the outer membrane of the *S. typhimurium* ECA-trace strain might also provide information as to the nature of the outer membrane defect (Smit et al., 1975). This analysis would also indicate if a general

disruption of synthesis or translocation of outer membrane components is occurring in ECA-trace strains. As mentioned previously, the observation of "mixed" LPS-chemotypes in the ECA-trace strain suggested that lipid II may be interfering with glycosyltransferase activities of LPS synthesis. Lipid II accumulation may similarly affect synthesis of other outer membrane components.

A FAB/MS/MS analysis of purified preparations of ECA_{PL} might detect any side chain components that could represent cross-links. Previous studies on the chemical composition of ECA have reported small amounts of protein or amino acids associated with the preparations (Mayer and Schmidt, 1979). However the exact nature of these amino acids was not revealed. A FAB/MS/MS analysis would also verify that ECA_{PL} is pyrophosphate linked. In addition, analysis of purified peptidoglycan preparations might reveal the nature of the ECA-peptidoglycan association if such an association does exist.

The defect due to lipid II is unique to *S. typhimurium* as *E. coli* lipid II accumulators are not unusually sensitive to hydrophobic agents (Meier-Dieter et al., 1990). An analysis of the lipid II isolated from *E. coli* to determine if lipids IIa and IIb are present might be informative. The absence of lipids IIa and IIb in an *E. coli* lipid II accumulating strain would implicate these compounds in the hydrophobic sensitivity phenotype of the *S. typhimurium* ECA-trace strain.

rfe Dependent O-Antigen Synthesis

Recent experimental evidence has identified the *rfe* gene product as the UDP-GlcNAc: undecaprenylphosphate GlcNAc-1-phosphate transferase (GlcNAc-P transferase) responsible for synthesis of lipid I (Meier-Dieter et

al., 1990; Meier-Dieter et al., 1992). However, previous reports of *rfe*-dependent O8 and O9 antigen synthesis have been shown to involve a Glc-pyrophosphorylundecaprenol carrier (Weisgerber and Jann, 1982). These studies imply that the *rfe* gene product is a UDP-Glucose: undecaprenylphosphate Glucose-1-phosphate transferase (Glucose-P transferase). Furthermore, the discrepancy is further complicated by the observation that all *rfe* mutations studied to date abolish both ECA and O8 or O9 synthesis (Mäkelä et al., 1970; Mäkelä and Mayer, 1974; Meier-Dieter et al., 1990) and these mutations are all complemented by a single open reading frame (Meier-Dieter et al., 1992; Ohta et al., 1991). In one of these studies it was also shown that the in-vivo effect of tunicamycin on ECA synthesis was decreased by the increased expression of the *rfe* gene on a multicopy plasmid (Meier-Dieter et al., 1992). This result again suggested that *rfe* is the structural gene for the GlcNAc-P transferase. It seems unlikely that a transferase enzyme would have such broad specificity allowing transfer of two different sugar-1-phosphates, glucose-1-phosphate and GlcNAc-1-phosphate, from their respective nucleotide carriers to undecaprenyl monophosphate. Previous explanations for the role of *rfe* in both O-antigen and ECA synthesis suggested that Rfe might be a unique lipid carrier or might function in the structural modification of the undecaprenol molecule (Mäkelä and Mayer, 1974). However, as mentioned above, the mass spectrum of lipid II established that undecaprenol is indeed the lipid carrier and that its structure is not modified (Figure 47). It has also been proposed that Rfe is a possible compartmentalization factor, helping to sequester in close proximity factors common to both ECA and *rfe*-dependent O-antigen synthesis (Mäkelä and Mayer, 1974). Finally, it has been suggested that *rfe* is a regulatory gene which functions in some unknown manner for the

synthesis of both ECA and *rfe*-dependent O-antigens (Ohta et al., 1991).

The tunicamycin sensitivity of O8 synthesis in strain HR193 (Figure 51) strongly supports the conclusion that the GlcNAc-P transferase is indeed the product of the *rfe* gene. This follows from establishment of tunicamycin as a specific inhibitor of reactions involved in the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to polyprenol phosphate acceptors (Takatsuki et al., 1975; Tkacz and Lampen, 1975; Mahoney and Duskin, 1979). The tunicamycin sensitivity of both ECA (Figure 50) and O8 (Figure 51) synthesis indicates that GlcNAc-P transferase is involved in both biosynthetic processes. However, to clarify the role of GlcNAc-PP-C_{55} rather than Glucose-PP-C_{55} in O8 synthesis we must briefly review pertinent aspects of earlier investigations involved with determining the mechanism of O8 and O9 biosynthesis.

The earliest work involved attempts to isolate intermediates involved in O8 and O9 side chain synthesis from phenol or butanol extracts of in vitro reactions (Flemming and Jann, 1978; 1978a). These extracts were stated to contain lipid-carrier-bound forms that were water-soluble. The reducing terminal sugar of these intermediates was identified as glucitol (sorbitol) by paper-chromatography after borohydride reduction and hydrolysis. Unfortunately sorbitol and mannitol were the only polyol standards run in their chromatography system; the migration of glucosaminitol (acid hydrolysis would have removed the N-acetyl group from GlcNAc and the researchers did not perform an N-acetylation to reacetylate before chromatography analysis) in this system is not known. In contrast, the lipid-linked O8 material isolated from HR193 (*man*A, *pgi* O8) grown in the absence of glucose was insoluble in butanol, as well as several other solvent systems and aqueous solutions without detergent. It was clear that we were

working with the carrier form of the O8 polymer due to its mild acid lability (Figure 51). Furthermore, the "carrier form" of O8 material isolated in this study reacted with O8 antibody (Figure 52), and it was synthesized in a *pgi* background in the total absence of glucose (Figures 51 and 52). The resistance of the isolated material to alkali (Figure 51) indicates that a sugar having a hydroxyl group in the 2-position (i.e. glucose or mannose) cannot be linked to pyrophosphate in the reducing terminal position. Collectively, this data indicates that glucose is not involved in the synthesis of O8 side-chains since the *pgi* mutation would preclude synthesis of the polymer. The alkali resistance of the polymer not only eliminates involvement of glucose-PP-C_{55} in O8 synthesis, but it also indicates that mannose-PP-C_{55} is not involved either. Jann et al., may have been led into believing O8 synthesis was glucose dependent as a result of their earlier observations that suggested increased in-vitro incorporation of mannose into membrane-bound material using membranes prepared from cells grown in the presence of 0.5% glucose (Kanegasaki and Jann, 1979). Subsequent in vitro studies by Jann and coworkers also suggested a role for glucose-PP-C_{55} in O8 and O9 side-chain synthesis using membranes isolated from *rfe* mutants (Jann et al., 1982; Weisgerber and Jann, 1982). However, in these in vitro reactions, UDP-glucose concentrations of 10mM were required to observe mannose incorporation from GDP-mannose into O8 or O9 polymer. Based on these observations, these investigators identified the acceptor of mannose residues as Glc-pyrophosphorylundecaprenol (Glucose-PP-C_{55}). In contrast, we were unable to detect any UDP-GlcNAc or UDP-glucose dependent incorporation of mannose into polymer in a similar in vitro system using membranes from our strains. However, we did observe some in-vitro incorporation of mannose from GDP-mannose into the insoluble membrane fraction (Results).

This is probably due to transfer of mannose to existing lipid I present in the membrane as it was not affected by added tunicamycin, UDP-glucose or UDP-GlcNAc. This material was insoluble in butanol and remained in the residue after extraction by water and two organic solvent systems. The nature of the mannose containing product observed by Jann's group is uncertain since it has been repeatedly shown that *rfe* mutations abolish all O8 and related O-antigen synthesis (Meier-Dieter et al., 1990; 1992). It is possible that the high concentration of UDP-glucose employed by these investigators may have forced a defective *rfe*-product to catalyze the synthesis of some Glc-PP-C_{55}. Indeed, using synthetic Glc-PP-C_{55} and mannosyl-1-3-Glc-PP-C_{55} as acceptors of mannose in their *rfe*-membrane system, Jann and coworkers were able to show mannose incorporation into polymer from GDP-mannose (Jann et al., 1985). In this study the incorporation of mannose into polymer from GDP-mannose utilized only 25% of the Glc-PP-C_{55} acceptor while incorporation was near 100% using mannosyl-1-3-glucosylpyrophosphoryl-undecaprenol as acceptor. The reduced transfer of mannose to Glc-PP-C_{55} may reflect that this compound is not the preferred acceptor for the first mannosyl transferase. In fact, a certain amount of cross-specificity in the mannosyl transferases can be inferred from a *E. coli* O9-*rfb* deletion study (Kido et al., 1989). In this study, deletion of the 1-3 mannosyl transferase structural gene produced a new O-antigen lacking mannose residues linked 1-3. The remaining mannosyl transferase(s) were able to synthesis a mannose polymer linked 1-2 and apparently did so by linking together trisaccharide subunits (Kido et al., 1989). Extrapolating from Kido's study, nonspecificity of the first mannosyl transferase might allow the possibility of transfer of mannose from GDP-mannose with a reduced reaction (25%) rate to Glc-PP-C_{55}.

Corroboration for the role of Rfe as the GlcNAc-P transferase and the involvement of lipid I in *rfe*-dependent O8 side-chain synthesis was provided in this work by the identification of GlcNAc and N-acetyl-glucosaminitol in unreduced and reduced hydrolysates of the partially purified O8 polymer material, respectively, in samples analyzed by the Dionex BIO-LC carbohydrate analysis system (Figures 55 &56). Glucose and sorbitol were absent in these samples. Addition of N-acetyl-glucosaminitol to the reduced O8 sample resulted in an increase in the intensity of the peak identified as N-acetyl-glucosaminatol (Figure 57). Moreover, addition of sorbitol to the reduced O8 sample produced a separate peak distinct from that identified as N-acetyl-glucosaminitol (Figure 58).

Accordingly, it is concluded that lipid I is specifically involved in O8 antigen synthesis. The results of these studies also support previous data (Meier-Dieter et al., 1990; 1992) which identified the *rfe* gene product as a UDP-GlcNAc: undecaprenylphosphate GlcNAc-1-phosphate transferase.

However, the identification of GlcNAc at the reducing end of the O8 polymer and the sensitivity of O8 synthesis to tunicamycin still has not ruled out the possibility that the *rfe* gene product has a regulatory function. The increased resistance of ECA synthesis to tunicamycin in *E. coli* strains containing multiple copies of the *rfe* gene (Meier-Dieter et al., 1992) could be interpreted as a regulatory mechanism. One interpretation would be that the *rfe* gene product serves as a positive regulator similar to the heat-shock proteins sigma factor. In this case the *rfe* gene product enhances the RNA polymerase recognition of an as yet unidentified structural gene for the UDP-GlcNAc: undecaprenylphosphate GlcNAc-1-phosphate transferase increasing transcription. Another interpretation would have the *rfe* gene product interacting directly with the enzyme as a positive allosteric modulator. In

either model the absence of *rfe* would have to render the transferase gene or enzyme inactive to explain the ECA-minus phenotype of *rfe* mutants. Until the product of the *rfe* gene is isolated and assayed for UDP-GlcNAc: undecaprenylphosphate GlcNAc-1-phosphate transferase activity, an absolute identification of the function of this gene cannot be assigned.

LITERATURE CITED

Acker, G., D. Bitter-Suermann, U. Meier-Dieter, H. Peters, H. Mayer. 1986. Immunocytochemical localization of enterobacterial common antigen in *Escherichia coli* and *Yersinia enterocolitica* cells., J. Bacteriol. 168: 348-356

Acker, G., and C. Kammerer. 1990. Localization of enterobacterial common antigen immunoreactivity in the ribosomal cytoplasm of *Escherichia coli* cells cryosubstituted and embedded at low temperature. J. Bacteriol. 172: 1106-1113

Acker, G., G. Schmidt, H. Mayer. 1982. Accessibility of Enterobacterial Common Antigen to Antibodies in Encapsulated and non-capsulated S and R forms of *Escherichia coli* J. Gen. Microbiol. 128: 1577-1583

Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. p. 115-117. In E. F. Neufeld and V. Ginsburg (ed). Methods in Enzymology Vol. VIII. Complex Carbohydrates. Academic Press Inc. Orlando.

Ames, G. F.-L., E. N. Spudich, H. Nikaido. 1974. Protein composition of the outer membrane of *Salmonella typhimurium* Effect of lipopolysaccharide mutations. J. Bacteriol. 117: 406-416

Aoki, S., M. Merkel, W. R. McCabe. 1966. Immunofluorescent Demonstration of the Common Enterobacterial Antigen. P.N.A.S. 121: 230-234

Ashwell, G. 1966. New Colorimetric Methods of Sugar Analysis. p. 85-95. In E. F. Neufeld and V. Ginsburg (ed). Methods in Enzymology Vol. VIII. Complex Carbohydrates. Academic Press Inc. Orlando.

Bachmann, B. J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K12. Bacteriol. Rev. 36: 525-557

Barr, K., P. Nunes-Edwards, P. D. Rick. 1989. In vitro synthesis of a lipid-linked trisaccharide involved in synthesis of enterobacterial common antigen. J. Bacteriol. 171: 1326-1332

Barr, K., and P. D. Rick. 1987. Biosynthesis of enterobacterial common antigen in *Escherichia coli* In vitro synthesis of lipid-linked intermediates. J. Biol. Chem. 262: 7142-7150

Barr, K., S. Ward, U. Meier-Dieter, H. Mayer, P. D. Rick. 1988. Characterization of an *Escherichia coli rff* mutant defective in transfer of N-acetylmannosaminuronic acid (ManNAcA) from UDP-ManNAcA to a lipid-linked intermediate involved in enterobacterial common antigen synthesis. J. Bacteriol. 170: 228-233

Barry, A. L., and C. Thornsberry. 1980. Appendix 2. Approximate minimal inhibitory concentrations of more susceptible strains among common bacterial pathogens. p. 498-499. In E. H. Lennette (ed.). Manual of Clinical Microbiology, 3rd Ed., Amer. Soc. for Microbiol., Washington D.C.

Bayer, M. H. 1979. The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. p.167-202. In Masayori Inouye (ed.), Bacterial Outer Membranes, John Wiley & Sons Inc., New York.

Bayer, M. H., G. P. Costello, M. E. Bayer. 1982. Isolation and partial characterization of membrane vesicles carrying markers of the membrane adhesion sites. J. Bacteriol. 149: 758-767

Behrens, N. H., and E. Tabora. 1978. Dolichol intermediates in the glycosylation of proteins. p. 402-435. In V. Ginsburg (ed.). Methods Enzymol. 50. Academic Press, New York.

Brahmbhatt, H. N., N. B. Quigley, P. R. Reeves. 1986. Cloning part of the region encoding biosynthetic enzymes for surface antigen (O-antigen) of *Salmonella typhimurium*. Mol. Gen. Genet. 203: 172-176

Brahmbhatt, H. N., P. Wyk, N. B. Quigley, P. R. Reeves. 1988. Complete physical map of the *rfb* gene cluster encoding biosynthetic enzymes for the O antigen of *Salmonella typhimurium* LT2. J. Bacteriol. 170: 98-102

Braun, V., and K. Rehn. 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of *E. coli* cell envelope. J. Bacteriol. 114:1264-1270.

Bullas, L. R., and J.-I. Ryu. 1983. *Salmonella typhimurium* LT2 Strains Which are r- m+ for all three chromosomally located systems of DNA restriction and modification. J. Bacteriol. 156: 471-474

Chai, T.-J., and J. Foulds. 1977. *Esherichia coli* K-12 *tolF* mutants: alterations in protein composition of the outer membrane. J. Bacteriol. 130: 781-786

Clark, D. 1984. Novel antibiotic hypersensitive mutants of *Esherichia coli* genetic mapping and chemical characterization. FEMS Microbiol. Lett. 21: 189-195

Coleman, W. G., and L. Leive. 1979. Two mutations which affect the barrier function of the *Esherichia coli* K-12 outer membrane. J. Bacteriol. 139:899-910

Creeger, E. S., T. Schulte, L. I. Rothfield. 1984. Regulation of membrane glycosyltransferases by the *sfrB* and *rfaH* genes of *Escherichia coli* and *Salmonella typhimurium*. J. Biol. Chem. 259: 3064-3069

Dankert, M., A. Wright, W. S. Kelley, P. W. Robbins. 1966. Isolation, Purification, and properties of the lipid-linked intermediates of O-antigen biosynthesis. Arch. of Biochem. Biophys. 116: 425-435

Davis, R. W., D. Botstein, J. R. Roth. 1980. Advanced Bacterial Gentics. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Dell, A., J. Oates, C. Lugowski, E. Romanowska, L. Kenne, B. Lindberg. 1984. The enterobacterial common-antigen. A cyclic polysaccharide. Carbohydrate Res. 133: 95-104

Domingue, G., and E. Johnson. 1974. Isolation of subcelular Fractions Containing Immunogenic Enterobacterial Common Antigen. Z. Immun.-Forsch. 148: 23-38

Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350-356

Emmerling, G., U. Henning, T. Guilik-Krzywicki. 1977. Order-disorder conformational transition of hydrocarbon chains in lipopolysaccharides from *Escherichia coli* Eur. J. Biochem. 78: 503-509

Flemming, H.-C., and K. Jann. 1978. Biosynthesis of the 08-antigen of *Escherichia coli* Glucose at the reducing end of the polysaccharide and growth of the chain. FEMS Microbiol. Lett. 4: 203-205

Flemming, H.-C., and K. Jann. 1978. Biosynthesis of the 09 antigen of *Escherichia coli* Growth of the polysaccharide chain. Eur. J. Biochem. 83: 47-52

Fraenkel, D. G., and B. L. Horecker. 1964. Pathways of D-Glucose Metabolism in *Salmonella typhimurium*. A study of a mutant lacking phosphoglucose isomerase. J. Biol. Chem. 239: 2765-2771

Fraenkel, D. G., and R. T. Vinopal. 1973. Carbohydrate Metabolism in Bacteria. Annu. Rev. Microbiol. 27: 69-100

Gabriel, O. 1987. Biosynthesis of sugar residues for glycogen, peptidoglycan, lipopolysaccharide, and related systems. pp.504-511 In F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella Typhimurium*. Cellular and Molecular Biology., Vol. 1. American Society For Microbiology, Washington, D. C.

Galanos, C. O., and E. T. Luderitz. 1975. Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. 54: 603-610

Galanos, C. O., E. T. Luderitz, E. T. Rietschel, O. Westphal. 1977. Newer aspects of the chemistry and biology of bacterial lipopolysaccharides with special reference to their lipid A component. p. 239-335. In T. W. Goodwin (ed.). International Review of Biochemistry, Vol.14. Biochemistry of Lipids II, University Park Press, Baltimore.

Galanos, C., O. Luderitz, O. Westphal. 1969. A New Method for the Extraction of R Lipopolysaccharides. Eur. J. Biochem. 9: 245-249

Gavan, T. L., and A. L. Barry. 1980. Microdilution Test Procedures. p. 459-462. In E. H. Lennette (ed.). Manual of Clinical Microbiology, 3rd Ed., Amer. Soc. for Microbiol., Washington D.C.

Glauert, A. M., and M. J. Thornley. 1969. The topography of the bacterial cell wall. Annu. Rev. Microbiol. 23:159-198.

Grogan, D. W., and J. E. Cronan. 1984. Genetic characterization of the *Escherichia coli* cyclopropane fatty acid (*cfa*) locus and neighboring loci. Mol. Gen. Genet. 196: 367-372

Grundstrom, T., S. Normark, K.-E. Magnusson. 1980. Overproduction of outer membrane protein suppresses *envA*-induced hyperpermeability. J. Bacteriol. 144: 884-890

Hardy, M. R., and R. R. Townsend. 1988. Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection. P.N.A.S. 85: 3289-3293

Higashi, Y., J. L. Strominger, C. C. Sweeley. 1967. Structure of a lipid intermediate in cell wall peptidoglycan synthesis: A derivative of a C55 isoprenoid alcohol. P.N.A.S. 57: 1878-1884

Higashi, Y., J. L. Strominger, C. C. Sweeley. 1970. Biosynthesis of the peptidoglycan of bacterial cell walls. XXI. isolation of free C55-isoprenoid alcohol and of lipid intermediates in peptidoglycan synthesis from *Staphylococcus aureus*. J. Biol. Chem. 245: 3697-3702

Hirota,Y., H. Suzuki, Y. Nishimura, S. Yasuda. 1977. On the process of cellular division in *Escherichia coli*: a mutant of *E. coli* lacking a murein-lipoprotein. Proc. Natl. Acad. Sci. U.S.A. 74: 1417-1420

Inouye, Masayori. 1979. What is the Outer Membrane?, p.1-14. In Masayori Inouye (ed.), Bacterial Outer Membranes, John Wiley & Sons Inc., New York.

Jacob, F., and E. L. Wollman. 1961. Sexuality and the genetics of bacteria. Academic Press, New York.

Jann, K., G. Goldemann, C. Weisgerber, C. Wolf-Ullisch, S. Kanegasaki. 1982. Biosynthesis of the O9 antigen of *Escherichia coli* Initial reaction and overall mechanism. Eur. J. Biochem. 127: 157-164

Jann, K., M. Pillat, C. Weisgerber, V. N. Shibaev, V. I. Torgov. 1985. Biosynthesis of the O9 antigen of *Escherichia coli* Synthetic glycosyldiphosphomoraprenols as for requirement of mannose acceptors. Eur. J. Biochem. 151: 393-397

Jiang, X.-M., B. Neal, F. Santiago, S. J. Lee, L. K. Romana, P. R. Reeves. 1991. Structure and sequence of the *rfb* (O antigen) gene cluster of *Salmonella* serovar *typhimurium* (strain LT2). Mol. Microbiol. 5: 695-713

Johnson, E. J., G. J. Domingue, G. D. Klein. 1976. Anatomical Locus of the Common Enterobacterial Antigen. Z. Immun.-Forsch. 152: 15-26

Johnson, G. L., J. H. Hoger, J. H. Ratnayake, J. S. Anderson. 1984. Characterization of three intermediates in the biosynthesis of teichurronic acid of *Miccrococcus luteus*. Arc. Biochem. Biophys. 235: 679-691

Kamio, N., and H. Nikaido. 1976. Outer membrane of *Salmonella typhimurium*: Accessibility of phospholipid head groups to phospholipase C and cyanogen bromide activated dextran in the external medium. Biochemistry 15: 2561-2570

Kanegasaki, S, and K. Jann. 1979. Demonstration by membrane reconstitution of a butanol-soluble intermediate in the biosynthesis of the O9 antigen of *Escherichia coli* Eur. J. Biochem. 95: 287-293

Kido, N., M. Ohta, K.-I. Iida, T. Hasegawa, H. Ito, Y. Arakawa, T. Komatsu, N. Kato. 1989. Partial deletion of the cloned *rfb* gene of *Escherichia coli* O9 results in synthesis of a new O-antigenic lipopolysaccharide. J. Bacteriol. 171: 3629-3633

Kitaoka, M., H. Nagaki, T. Kinoshita, M. Kurabayashi, T. Koyama, K. Ogura. 1990. Negative ion fast atom bombardment-tandem mass spectrometry for structural analysis of isoprenoid diphosphates. Analyt. Biochem. 185: 182-186

Kopmann, H. J., and K. Jann. 1975. Biosynthesis of the O9 antigen of *Escherichia coli* The polysaccharide component of *E. coli* O9:K29⁻. Eur. J. Biochem. 60: 587-601

Kuhn, H.-M., G. Adamus, E. Romanowska, H. Mayer. 1981. Effects of proteins on the immunogenicity of enterobacterial common antigen. Infect. Immun. 34: 373-377

Kuhn, H.-M., S. Basu, H. Mayer. 1987. Comparison of enterobacterial common antigen from different species by serological techniques. Eur. J. Biochem. 162: 69-74

Kuhn, H.-M., and H. Mayer. 1987. Occurrence of LPS-bound enterobacterial common antigen (ECA) in *E. coli* K-12. International Conference on Endotoxins, Amsterdam II. Abstract 88. May 22-23, Amsterdam

Kuhn, H.-M., U. Meier-Dieter, H. Mayer. 1988. ECA, the enterobacterial common antigen. FEMS Microbiol. Rev. 54: 195-222

Kunin, C. M., M. V. Beard, N. E. Halmagyi. 1962. Evidence for a common hapten associated with endotoxin fractions of *E. coli* and other enterobacteriaceae. Proc. Soc. Exp. Biol. Med. 111:160-166

Kuo, T.-T., and B. A. D. Stocker. 1970. ES18, a general Transducing Phage for smooth and nonsmooth *Salmonella typhimurium*. Virology 42: 621-632

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Leive, L. 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli* Biochem. Biophys. Res. Commun. 21: 290-296

Leive, L. 1974. The barrier function of the gram-negative envelope. Ann. N. Y. Acad. Sci. 235:109-127

Leive, L., S. Telesetsky, W. G. Coleman, D. Carr. 1984. Tetracycline of various hydrophobicities as a probe for permeability of *Escherichia coli* outer membrane. Antimicrob. Agents Chemother. 25: 539-544

Le Minor, L., A.-M. Chalon, M. Veron. 1972. Recherches sur la presence de l'antigene commun des <<enteroacteriaceae>> (antigene Kunin) chez les <<Yersinia>>, <<Levinea>>, <<Aeromonas>>, et <<Vibrio>>. Ann. Inst. Pasteur (Paris) 123: 761-774

Lew, H. C., P. H. Mäkelä, H.-M. Kuhn, H. Mayer, H. Nikaido. 1986. Biosynthesis of enterobacterial common antigen requires dTDPglucose pyrophosphorylase determined by a *Salmonella typhimurium rfb* gene and a *Salmonella montevideo rfe* gene. J. Bacteriol. 168: 715-721

Lew, H. C., H. Nikaido, P. H. Mäkelä. 1978. Biosynthesis of uridine diphosphate N-acetylmannosaminuronic acid in *rff* mutants of *Salmonella typhimurium*. J. Bacteriol. 136: 227-233

Lindberg, A. A., and C.-G. Hellerqvist. 1980 Rough mutants of *Salmonella typhimurium*: Immunochemical and structural analysis of lipopolysaccharides from *rfaH* mutants. J. Gen. Microbiol. 116: 25-32

Luderitz, O., O. Westphal, A. M. Staub, H. Nikaido. 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides, p. 145-233. In G. Weinbaum, S. Kadis, S. L. Ajl (ed.), Microbial Toxins, Vol. 4. Bacterial Endotoxins. Academic Press, Inc., New York.

Lugowski, C., M. Kulakowska, E. Romanowska. 1983. Enterobacterial common antigen-tetanus toxoid conjugate as immunogen. Infect. Immun. 42: 1086-1091

Lugowski, C., E. Romanowska, L. Kenne, B. Lindberg. 1983. Identification of a trisaccharide repeating-unit in the enterobacterial common-antigen. Carbohydrate Res. 118: 173-181

Lugtenberg, B., and L. van Alphen. 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta. 737: 51-115

Maeland, J. A., and A. Digranes. 1975. Common enterobacterial antigen in *Yersinia enterocolitica*. Acta Pathol. Microbiol. Scand. Sect. B. 83: 382-386

Mahoney, W. C., and D. Duskin. 1979. Biological activities of the two major components of tunicamycin. J. Biol. Chem. 254: 6572-6576

Mäkelä, P. H., M. Jahkola, O. Luderitz. 1970. A new gene cluster rfe concerned with the biosynthesis of *Salmonella* lipopolysaccharide. J. Gen. Microbiol. 60: 91-106

Mäkelä, P. H., and H. Mayer. 1974. Participation of lipopolysaccharide genes in the determination of the enterobacterial common antigen: Analysis in *Salmonella* groups B and C1. J. Bacteriol. 119: 765-770

Mäkelä, P. H., H. Mayer, H. Y. Whang, E. Neter. 1974. Participation of lipopolysaccharide genes in the determination of the enterobacterial common antigen: analysis of R mutants of *Salmonella minnesota*. J. Bacteriol. 119: 760-764

Mäkelä, P. H., G. Schmidt, H. Mayer, H. Nikaido, H. Y. Whang, E. Neter. 1976. Enterobacterial common antigen in *rfb* deletion mutants of *Salmonella typhimurium*. J. Bacteriol. 127: 1141-1149

Maloy, S. R., W. D. Nunn.1961. Selection for the Loss of Tetracycline Resistance by *Escherichia coli* J. Bacteriol. 145: 1110-1112

Maniatis, T., E. F. Fritsch, J. Sambrook. 1982 Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Mannel, D., and H. Mayer. 1978. Isolation and chemical characterization of the enterobacterial common antigen. Eur. J. Biochem. 86: 361-370

Mayer, H., G. Schmidt. 1979. Chemistry and biology of the enterobacterial common antigen (ECA). Curr. Top. Microbiol. Immunol. 85: 99-153

McKenney, K., H. Shimatake, D. Court, U. Schmeissner, C. Brady, M. Rosenberg. 1981. A System to study promoter and terminator signals recognized by *Escherichia coli* RNA polymerase. pp. 383-415 In J. Chirikjian, T. Papas, (ed). Gene Amplification and Analysis. Vol 2. Elsevier/North-Holland pub. Co. N.Y

Meier-Dieter, U., G. Acker, H. Mayer. 1989. Detection of enterobacterial common antigen on bacterial cell surfaces by colony-immunoblotting: effect of its linkage to lipopolysaccharide. FEMS Microbiol. Lett. 59: 215-220

Meier-Dieter, U., K. Barr, R. Starman, L. Hatch, P. D. Rick. 1992. Nucleotide sequence of the *Escherichia coli rfe* gene involved in the synthesis of enterobacterial common antigen. Molecular cloning of the *rfe-rff* gene cluster. J. Biol. Chem. 267: 746-753

Meier-Dieter, U., R. Starman, K. Barr, H. Mayer, P. D. Rick. 1990. Biosynthesis of enterobacterial common antigen in *Escherichia coli* Biochemical characterization of Tn10 insertion mutants defective in enterobacterial common antigen synthesis. J. Biol. Chem. 265 13490-13497

Meier, U., H. Mayer. 1985. Genetic location of genes encoding enterobacterial common antigen. J. Bacteriol. 163: 756-762

Meier, U., H. Mayer, D. Bitter-Suermann. 1986. A blot-immunobinding assay for detection of LPS-linked enterobacterial common antigen. EOS-Riv-Immunol. VI, 3/Supplement 166

Michel, G. 1979. The changes in cell size and phospholipid composition during growth of a chain-forming *envC* mutant of *Escherichia coli* FEBS Lett. 100: 258-260

Michel, G., D. Disavino, J. Starka. 1977. Phospholipid composition and phenotypic correction of an *envC* division mutant of *Escherichia coli* J. Bacteriol. 129: 145-150

Misra, R., and S. A. Benson. 1988. Genetic Identification of the pore domain of the ompC porin of *Escherichia coli* K12. J. Bacteriol. 170: 3611-3617

Mizuno, T., and M. Kageyama. 1978. Separation and characterization of the outer membrane of *Pseudomonas aeruginosa* J. Biochem. (Tokyo) 84: 179-191

Muhlradt, P.F., and J. R. Golecki. 1975. Asymmetrical distribution and artifactual reorientation of lipopolysaccharide in the outer membrane bilayer of *Salmonella typhimurium*. Eur. J. Biochem. 51: 343-352

Mulford, C. A., and M. J. Osborn. 1983. An intermediate step in translocation of the lipopolysaccharide to the outer membrane of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. 80: 1159-1163

Munford, R. S., and C. L. Hall. 1986. Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophile enzyme. Science 234: 203-205

Nakae, T, and H. Nikaido. 1971. Multiple Molecular Forms of Uridine Diphosphate Glucose Pyrophosphorylase from *Salmonella typhimurium*. I. Catalytic Properties of Various Forms. J. Biol. Chem. 246: 4386-4396

Nakae, T, and H. Nikaido. 1971a. Multiple Molecular Forms of Uridine Diphosphate Glucose Pyrophosphorylase from *Salmonella typhimurium*. II. Genetic determination of Multible Forms. J. Biol. Chem. 246: 4397-4403

Nakamura, H., and A. Suganuma. 1972. Membrane mutation associated with sensitivity to acriflavine in *Escherichia coli* J. Bacteriol. 110: 329-335

Nakamura, K., and M. Inouye. 1979. Homology of the gene coding for outer membrane lipoprotein within various gram-negative bacteria. J. Bacteriol. 137: 595-604

Neidhardt, F. C. 1987. Chemical Composition of *Escherichia coli*. pp. 3-6 In F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella Typhimurium*. Cellular and Molecular Biology., Vol. 1. American Society For Microbiology, Washington, D. C.

Nikaido, H. 1976. Outer membrane of *Salmonella typhimurium* transmembrane diffusion of some hydrophobic substances. Biochimica et Biophysica Acta. 433: 118-132

Nikaido, H., M. Levinthal, K. Nikaido, K. Nakane. 1967. Extended deletions in the histidine-rough-B region of the *Salmonella* chromosome. Proc. Natl. Acad. Sci. U. S. A. 57: 1825-1832

Nikaido, H., K. Nikaido, P. H. Mäkelä. 1966. Genetic determination of enzymes synthesizing O-specific sugars of *Salmonella* lipopolysaccharides. J. Bacteriol. 91: 1126-1135

Nikaido, H., Y. Takeuchi, S. Ohnishi, T. Nakae. 1977. Outer membrane of *Salmonella typhimurium*. Electron spin resonance studies. Biochim. Biophys. Acta 465:152-164

Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49:1-32.

Nnalue, N. A., and B. A. D. Stocker. 1987. The effects of O-antigen character and enterobacterial common antigen content on the in vivo persistence of aromatic-dependent *Salmonella* sp. live-vaccine strains. Microb. Pathogen. 3: 31-44

Normark, S., H. G. Boman, G. D. Bloom. 1971. Cell division in a chain-forming *envA* mutant of *Escherichia coli* K12. Acta Path. Microbiol. Scand. Sec. B. 79: 651-664

Normark, S., H. G. Boman, E. Matsson. 1969. Mutant of *Escherichia coli* with anomalous cell division and ability to decrease episomally and chromosomally mediated resistance to ampicillin and several other antibiotics. J. Bacteriol. 97: 1334 -1342

Novel, G., and M. Novel. 1973. Mutants d' *Escherichia coli* K12 affectes pour leur croissance sur methyl-ß-D-glucuronide: localisation du gene de structure de la ß--D-glucuronidase (*uidA*). Mol. Gen. Genet. 120: 319-335

Ohta, M., K. Ina, K. Kusuzaki, Y. Arakawa, N. Kato. 1991. Cloning and expression of the *rfe-rff* gene cluster of *Escherichia coli* Mol. Microbiol. 5: 1853-1860

Osborn, M. J., J. E. Gander, E. Parisi, J. Carson. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of the cytoplasmic and outer membrane. J. Biol. Chem. 247: 3962-3872

Osborn, M. J., and R. Munson. 1974. Separation of the inner (cytoplasmic) and outer membranes of gram-negative bacteria. p. 642-653. In S. Fleischer, and L. Packer (ed.), Methods in Enzymology, vol. 31. Academic Press, Inc., New York

Ostrovsky de Spicer, P., K. O'Brien, S. Maloy. 1991. Regulation of the Proline Utilization in *Salmonella typhimurium*, a membrane-associated dehydrogenase binds DNA In Vitro. J. Bacteriol. 173: 211-2193

Park, J. T. 1987. Murein synthesis. pp.663-671 In F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella Typhimurium*. Cellular and Molecular Biology., Vol. 1. American Society For Microbiology, Washington, D. C.

Park, J. T., and M. J. Johnson. 1979. A submicrodetermination of glucose. J. Biol. Chem. 181: 149-151

Peters, H., M. Jurs, B. Jann, K. Jann, K. N. Timmis, D. Bitter-Suermann. 1985.Monoclonal antibodies to enterobacterial common antigen and to *Escherichia coli* lipopolysaccharide outer core: Demonstration of an antigenic determinant shared by enterobacterial common antigen and *E. coli* K5 capsular polysaccharide. Inf. & Immun. 50: 459-466

Raetz, C. R. H. 1986. Molecular genetics of membrane phospholipid synthesis. Annu. Rev. Genet. 29: 253-295

Raetz, C. R. H., and J. Foulds. 1977. Envelope composition and antibiotic hypersensitivity of *Escherichia coli* mutants defective in phosphatidylserine synthetase. J. Biol. Chem. 252: 5911-5915

Ramia, S., E. Neter, D. J. Brenner. 1982. Production of enterobacterial common antigen as an aid to classification of newly identified species of the families Enterobacteriaceae and Vibrionaceae. Int. J. Syst. Bacteriol. 32: 395-398

Rapin, A. M. C., and H. M. Kalckar. 1971. The Relation of Bacteriophage Attachment to Lipopolysaccharide Structure. p. 267-307 In G. Weinbaum, S. Kadis, S. J. Ajl (ed). Microbial Toxins,Bacterial Endotoxins, vol. 4. Academic Press, New York

Rayssiguier, C., D. S. Thaler, M. Radman. 1989. The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. Nature 342: 396-401

Reske, K., and K. Jann. 1972. The O8 antigen of *Escherichia coli* Structure of the polysaccharide chain. Eur. J. Biochem. 31: 320-328

Rick, P. D. 1987. Lipopolysaccharide biosynthesis. pp.648-662 In F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella Typhimurium*. Cellular and Molecular Biology., Vol. 1. American Society For Microbiology, Washington, D. C.

Rick, P. D., L. W.-M. Fung, C. Ho, M. J. Osborn. 1977. Lipid A mutants of *Salmonella typhimurium*. Purification and characterization of a lipid A precursor produced by a mutant in 3-deoxy-D-manno-octulosonate-8-phosphate synthetase. J. Biol. Chem. 252: 4904-4912

Rick, P. D., H. Mayer, B. A. Neumeyer, S. Wolski, D. Bitter-Suermann. 1985. Biosynthesis of enterobacterial common antigen. J. Bacteriol. 162: 494-503

Rick, P. D., B. A. Neumeyer, D. A. Young. 1983. Effect of altered lipid A synthesis on the synthesis of the OmpA protein in *Salmonella typhimurium*. J. Biol. Chem. 258: 629-635

Rick, P. D., S. Wolski, K. Barr, S. Ward, L. Ramsay-Sharer. 1988. Accumulation of a lipid-linked intermediate involved in enterobacterial common antigen synthesis in *Salmonella typhimurium* mutants lacking dTDP-glucose pyrophosphorylase. J. Bacteriol. 170: 4008-4014

Rick, P. D., and D. A. Young, 1982a. Isolation and characterization of a temperature-sensitive lethal mutant of *Salmonella typhimurium* that is conditionally defective in 3-deoxy-D-manno-octulosonate-8-phosphate synthesis. J. Bacteriol. 150: 447-455

Rick, P. D., and D. A. Young, 1982b. Relationship between cell death and altered lipid A synthesis in a temperature-sensitive lethal mutant of *Salmonella typhimurium* that is conditionally defective in 3-deoxy-D-manno-octulosonate-8-phosphate synthesis. J. Bacteriol. 150: 456-464

Rinno, J., J. R. Golecki, H. Mayer. 1980. Localization of the Enterobacterial Common Antigen: Immunogenic and Nonimmunogenic Enterobacterial Common Antigen-Containing *Escherichia coli*. J. Bacteriol. 41: 814-821

Rinno, J., J. Gmeiner, J. R. Golecki, H. Mayer. 1980a. Localization of the Enterobacterial Common Antigen: *Proteus mirabilis* and Its Various L-Forms. J. Bacteriol. 141: 822-827

Roantree, R. J., T.-T. Kou, D. G. MacPhee. 1977. The effect of defined lipopolysaccharide core defects upon antibiotic resistances of *Salmonella typhimurium*. J. Gen. Microbiol. 103: 223-234

Rodolakis, A., P. Thomas, J. Starka. 1973. Morphological mutants of *Escherichia coli*. Isolation and ultrastructure of a chain-forming *envC* mutant. J. Gen. Microbiol. 75: 409-416

Rowley, D. L., and R. E. Wolf. 1991. Molecular characterization of the *Escherichia coli* K-12 *zwf* gene encoding glucose-6-phosphate dehydrogenase. J. Bacteriol. 173: 968-977

Sambrook, J., E. F. Fritsch, T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sanderson, K. E., T. MacAlister, J. W. Costerton, K.-J. Cheng. 1974. Permeability of lipopolysaccharide-deficient (rough) mutants of *Salmonella typhimurium* to antibiotics, lysozyme, and other agents. Can. J. Microbiol. 20: 1135-1145

Sanderson, K. E., H. Ross, L. Ziegler, P. H. Mäkelä. 1972. F+,Hfr, and F' Strains of *Salmonella typhimurium* and *Salmonella abony*. Bacteriol. Rev. 36: 608-637

Sanderson, K. E., and H. Saeed. 1972. Insertion of the F Factor into the Cluster of *rfa* (Rough A) genes of *Salmonella typhimurium*. J. Bacteriol. 112: 64-73

Schlecht, S., and G. Schmidt. 1969. Moglichkeiten zur differenzierung von *Salmonella*-R-formen mittles antibiotica und antibackterieller farbstoffe. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. 212:505-511

Schlecht, S., and O. Westphal,O. 1970. Investigations on the classification of *Salmonella*-R forms. 4. Communication. typing of *S. minnesota*-R-mutants by antibiotics. Zbl. Bakt. Parsit. Inf. Abt. Orig. 213: 356-381

Schindler, P. R. G., and M. J. Osborn. 1979. Interaction of divalent cations and polymyxin B with lipopolysaccharide. Biochemistry 18: 4425-4430

Schmidt, G., D. Mannel, H. Mayer, H. Y. Whang, E. Neter. 1976. The role of a lipopolysaccharide gene for immunogenicity of the enterobacterial common antigen. J. Bacteriol. 126: 579-586

Schmidt, G., H. Mayer, P. H. Mäkelä. 1976. Presence of *rfe* genes in *Escherichia coli*: Their participation in biosynthesis of O antigen and enterobacterial common antigen. J. Bacteriol. 127: 755-762

Shaw, M. K., J. L. Ingraham. 1965. Fatty acid composition of *Escherichia coli* as a possible controlling factor of the minimal growth temperature. J. Bacteriol. 90: 141-146

Smit, J., Y. Kamio, H. Nikaido. 1975. Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. 124: 942-958

Starkova, Z., N. Bonnaveiro, J. Starka. 1981. Hydrolysis of phospholipids by phospholipase C in intact cells of wild-type and envelope mutants of *Escherichia coli* K12. FEBS Lett 130: 261-264

Subbaiah, T. V., and B. A. D. Stocker. 1964. Rough mutants of *Salmonella typhimurium*. I. Genetics. Nature 201: 1298

Sukupolvi, S., and M. Vaara. 1989. *Salmonella typhimurium* and *Escherichia coli* mutants with increased outer membrane permeability to hydrophobic compounds. Biochimica et Biophysica Acta 988: 377-387

Sukupolvi, S., M. Vaara, I. M. Helander, P. Viljanen, P. H. Mäkelä. 1984. New *Salmonella typhimurium* mutants with altered outer membrane permeability. J. Bacteriol. 159: 704-712

Takatsuki, A., K. Kohno, G. Tamura. 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. Agr. Biol. Chem. 39: 2089-2091

Takayama, K., N. Qureshi, P. Mascagni. 1983. Complete structure of lipid A obtained from lipopolysaccharides of the heptoseless mutant of *Salmonella typhimurium*. J. Biol. Chem. 258: 12801-12802

Tkacz, J. S., and J. O. Lampen. 1975. Tunicamycin inhibition of polyisoprenyl N-acetylglucosaminyl pyrophosphate formation in calf-liver microsomes. Biochem. Biophys. Res. Commun. 65: 248-257

Valtonen, M. V., U. M. Larinkari, M. Plosila, V. V. Valtonen, P. H. Mäkelä. 1976. Effect of enterobacterial common antigen on mouse virulence of *Salmonella typhimurium*. Infect. and Immun. 13: 1601-1605

Van Alphen, L., B. Lugtenberg, R. von Boxtel, K. Verhoef. 1977. Architecture of the outer membrane of *Escherichia coli* K12. I. Action of phospholipases A2 and C on wild type strains and outer membrane mutants. Biochim. Biophys. Acta 466: 257-268

Van Driel, D. 1971. Cellular location of the lipoteichoic acids of *Lactobacillus fermenti* NCTC 6991 and *Lactobacillus casei* NCTC 6375. J. Ultrastruct. Res. 43: 483-497

Ward, J. B. 1981. Teichoic and Teichuronic Acids: Biosynthesis, Assembly, and Location. Microbiol. Rev. 45: 211-243

Washington II, J. A., and V. L. Sutter. 1980. Dilution Susceptibility Test: Agar and Macro-Broth Dilution Procedures. p. 453-458. In E. H. Lennette (ed.). Manual of Clinical Microbiology, 3rd Ed., Amer. Soc. for Microbiol., Washington D.C.

Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, N. Kleckner. 1984. New Tn10 derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene 32: 369-379

Weigand, R. A., and L. I. Rothfield. 1976. Genetic and physiological classification of periplasmic-leaky mutants of *Salmonella typhimurium*. J. Bacteriol. 125: 340-345

Weisgerber, C., and K. Jann. 1982. Glucosyldiphosphoundecaprenol, the mannose acceptor in the synthesis of the O9 antigen of *Escherichia coli* Eur. J. Biochem. 127: 165-168

Weiss, P. J., M. L. Andrew, W. W. Wright. 1957. Solubility of Antibiotics in Twenty-four Solvents: Use in Analysis. Antibiotics & Chemotherapy. 7: 374-377

Whang, H. Y., H. Mayer, G. Schmidt, E. Neter. 1982. Immunogenicity of the common enterobacterial antigen produced by smooth and rough strains. Infect. Immun. 6: 533-539

Whang, H. Y., and E. Neter. 1973. Production of common enterobacterial antigen by members of the family Enterobacteriaceae. Experientia 29: 96-97

Wilkinson, R. G., P. Gemski, B. A. D. Stocker. 1972. Non-smooth Mutants of *Salmonella typhimurium*: Differentiation by Phage Sensitivity and Genetic Mapping. J. Gen. Microbiol. 70: 527-554

Yamamori, S., N. Murazumi, Y. Araki, E. Ito. 1978. Formation and function of N-acetylglucosamine-linked phosphoryl- and pyrophosphorylundecaprenols in membranes from *Bacillus cereus*. J. Biol. Chem. 253: 6516-6522

Young, K., and L. L. Silver. 1991. Leakage of periplasmic enzymes from *envA*1 strains of *Escherichia coli* J. Bacteriol. 173: 3609-3614

Zarkowsky, H., and L. Glaser. 1969. The Mechanism of 6-Deoxyhexose Synthesis. The Purification of Deoxythymidine diphosphate-Glucose Oxidoreductase. J. Biol. Chem. 244: 4750-4756

Zinder, N. D. and J. Lederberg. 1952. Genetic exchange in *Salmonella* J. Bacteriol. 64: 679-699